Role of PP2C in cardiac lipid accumulation in obese rodents and its prevention by troglitazone

May-yun Wang and Roger H. Unger

Department of Internal Medicine, Touchstone Center for Diabetes Research, University of Texas Southwestern Medical Center, Dallas; and Veterans Affairs Medical Center, Dallas, Texas

Submitted 5 January 2004; accepted in final form 12 September 2004

Wang, May-yun, and Roger H. Unger. Role of PP2C in cardiac lipid accumulation in obese rodents and its prevention by troglitazone. Am J Physiol Endocrinol Metab 288:E216–E221, 2005. First published September 14, 2004; doi:10.1152/ajpendo.00004.2004.—In obese rodents, excess myocardial lipid accumulation (lipotoxicity) of myocardium may cause cardiomyopathy that in the obese Zucker diabetic fatty (ZDF) fa/fa rat can be prevented by treatment with troglitazone (TGZ). To determine the underlying mechanisms, we measured total 5'-AMP-activated kinase (AMPK) protein and its phosphorylated (P-AMPK) form in untreated ZDF fa/fa rat hearts, which lowering cardiac lipid content, increased P-AMPK. Expression of protein phosphatase 2C (PP2C), which inactivates AMPK activity by dephosphorylation, was increased in untreated ZDF fa/fa rat hearts, but fell with TGZ treatment, suggesting that PP2C can influence AMPK activity. In cultured mycardiocytes, fatty acids reduced P-AMPK, suggesting a feed-forward effect of lipid overload. Our findings highlight a role of PP2C and AMPK in the derangements of cardiac lipid metabolism in obesity and provide new insights as to the mechanisms of the liporegulatory disorder leading to lipotoxic cardiomyopathy.

MATERIALS AND METHODS

Animals. Male obese homozygous fa/fa Zucker diabetic fatty (ZDF) rats and their lean wild-type (+/+ ) littermates were bred in our laboratory from ZDF/Drt-fa (F10) rats, originally purchased from R. Peterson (University of Indiana School of Medicine, Indianapolis, IN). Male obese ob/ob mice and their wild-type control (+/+ ) mice (10–15 wk old) were obtained from the Jackson Laboratory (Bar Harbor, ME). All rats and mice received a standard laboratory chow (Teklad F6 8664; Teklad, Madison, WI) and tap water ad libitum.

Address for reprint requests and other correspondence: M.-y. Wang, Touchstone Center for Diabetes Research, Univ. of Texas Southwestern Medical Center, Dallas, TX 75390 (E-mail: May-yun.Wang@UTSouthwestern.edu).
TRIzol reagent (Life Technologies, Torrance, CA) following the RNA extraction protocol. For each RNA sample, reactions were carried out in triplicate using cDNA samples described above. Primers and probes were designed using Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA). The probe and primer sequences used were publicly available for the human PPARγ2 (NM_007475) as a housekeeping gene. The corresponding bands were quantified using NIH Image software (version 1.6; available at http://rsb.info.nih.gov/nih-image/).

Echocardiographic evaluation. Echocardiographic analysis of fa/fa ZDF rats was carried out as described (30).

RESULTS

Total and P-AMPK in the hearts of ZDF (fa/fa) rats and wild-type (+/+ controls). To compare the activity of AMPK in normal and lipid-laden hearts of untreated obese ZDF (fa/fa) rats, we measured the levels of total AMPK protein α-subunit (AMPKα) and active, phosphorylated AMPK (P-AMPKα; phosphorylated at Thr172 of the AMPK α-subunit). As shown in Fig. 1A, top and middle, the mean P-AMPKα level in these 14-wk-old, leptin-unresponsive obese fa/fa hearts was 67% lower than that of lean wild-type (+/+ ZDF controls (P < 0.0005). Total AMPK protein was slightly reduced in fa/fa compared with lean hearts. There were no significant changes in the mRNAs of AMPK α1-, α2 (Fig. 1A, bottom)-, β-, or γ-subunits (data not shown).

Total and P-AMPK in the hearts of ob/ob mice and wild-type (+/+ controls). To determine if a similar reduction in P-AMPKα was present in the hearts of another unleptinized rodent model, the leptin-deficient ob/ob mice, we compared them with their wild-type (+/+ controls). P-AMPKα was 81% lower in ob/ob mice (P < 0.0005; Fig. 1B, top and middle). There were no changes in the mRNAs of AMPK α1-, α2 (Fig. 1B, bottom)-, β-, or γ-subunits (data not shown). Thus our

Table 1. Sequences of TaqMan probes and primers and SYBR Green PCR primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>GenBank No.</th>
<th>Forward/Reverse Primer</th>
<th>TaqMan Probe (5′-Fam 3′-TAMRA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPKα1</td>
<td>U40819</td>
<td>TGAGAATGCAGCCCATCATTCT</td>
<td>ACGCTGCGGCGCCGACCC</td>
</tr>
<tr>
<td>AMPKα2</td>
<td>U12149</td>
<td>GCCGGGCGGACATGAGC</td>
<td>GCGCTGCGGCGCCGCGGAGCC</td>
</tr>
<tr>
<td>AMPKβ</td>
<td>U42411</td>
<td>ATGCGATGCCATCATTCT</td>
<td>CCGACACTGTCGAGGACCTCC</td>
</tr>
<tr>
<td>AMPKγ</td>
<td>X95578</td>
<td>TGCGCGGCGGCGGCGGAG</td>
<td>GAAAAGGGGCGGCGGAGGCGCTTTC</td>
</tr>
<tr>
<td>PPARγ</td>
<td>AF156666</td>
<td>CACCCAGCGGCGGCGGAGT</td>
<td>CACCCAGCGGCGGCGGAGG</td>
</tr>
<tr>
<td>36B4</td>
<td>NM_007475</td>
<td>CACCCAGCGGCGGCGGAGT</td>
<td>CACCCAGCGGCGGCGGAGG</td>
</tr>
<tr>
<td>PP2C*</td>
<td>J04503</td>
<td>TGGTTGCTGCTGCTGCTGCT</td>
<td>ACAGCAGCTGCTGCTGCTGCT</td>
</tr>
</tbody>
</table>

AMPK, 5′-AMP-activated kinase; PPAR, peroxisome proliferator-activated receptor; PP2C, protein phosphatase 2C.; SYBR Green PCR primers.
results suggest that the reduction of cardiac AMPK activity could be a consequence of congenital lack of leptin action.

Effects of glucose and FFAs on AMPK activity of myocardial cells. The lower cardiac AMPK activation could have been secondary to the metabolic abnormalities that constitute the ZDF (fa/fa) phenotype. Obese ZDF fa/fa and ob/ob rodents have higher plasma levels of glucose and FFA than their lean counterparts (13). To determine if the decreased AMPK activity in fa/fa and ob/ob hearts was secondary to the high concentrations of plasma glucose and/or FFA (in complexes with BSA), we examined the expression of AMPKα in the rat myocardial H9C2 cells cultured for 20 h with varying concentrations of these nutrients. There was no difference between P-AMPKα in cells cultured in 5 or 20 mM glucose (Fig. 2). However, in medium containing 5 mM glucose in the presence of 0.5 mM FFA–BSA or above, AMPKα protein and P-AMPKα were both greatly reduced (Fig. 2). This suggests that the elevation in intracellular fatty acids or their metabolites in the tissues of unleptinized rodents lowers cardiac AMPK activity and thus amplifies the consequences of a lipid surplus by interfering with their oxidation.

TGZ treatment of ZDF fa/fa rats restores cardiac AMPK activity. Because TGZ is effective in preventing lipid accumulation in the heart of ZDF (fa/fa) rats (30), we examined the effects of TGZ treatment on cardiac AMPK activity. Previously, we had found that in obese fa/fa rats TGZ lowered cardiac TG and ceramide content, preventing apoptosis and loss of contractile function (30). The rises in plasma FFA, TG, and glucose that occurred in untreated control rats were prevented by TGZ (30). The clinical and laboratory data obtained in the present study of untreated and TGZ-treated obese fa/fa rats are summarized in Table 2. Plasma glucose, FFA, and TG were significantly lower \( (P \leq 0.005) \) in TGZ-treated fa/fa rats than those of untreated animals, consistent with our earlier results. In addition, we found that, compared with untreated fa/fa ZDF rats, cardiac P-AMPKα of TGZ-treated fa/fa ZDF rats is increased four- to fivefold \( (P < 0.016) \) with only modest change in total AMPKα protein (Fig. 3A). There was no change in the mRNA levels of AMPK α1-, α2-, β-, and γ-subunits (data not shown). These results are consistent with the hypothesis that an increased AMPK activity contributes to the prevention of excess lipid accumulation in the fa/fa heart by TGZ.

Relationship of PP2C to changes in AMPK activity. PP2C is known to directly dephosphorylate and thus inactivate P-AMPKα in mammals (5, 17). Therefore, it seemed possible that the reduction in active P-AMPKα in untreated ZDF fa/fa rats could be a consequence of congenital lack of leptin action.
rats and its restoration toward near normal levels by TGZ treatment could be because of changes in this phosphatase. We therefore monitored the changes in cardiac PP2C mRNA and protein (Fig. 3, B and C) before and after TGZ treatment. PP2C expression was increased in the hearts of untreated ZDF rats compared with lean +/+ controls (Fig. 4A). After TGZ therapy, its mRNA and protein levels were decreased (P < 0.037 and 0.002, respectively; Fig. 3, B and C) concordantly with the rise in P-AMPKα levels (Fig. 3A). Similarly, administration to fa/fa rats for 2 mo of another PPARγ agonist, rosiglitazone, which was shown to reduce TG content in ob/ob hearts (20), led to a decrease in cardiac PP2C, an increase in P-AMPKα, and improved contractile performance in vivo (Table 3). These results are consistent with a role of PP2C in the change in cardiac AMPK activity in ZDF fa/fa rats. Because AMPK phosphorylation was also reduced in the hearts of obese ob/ob mice, we compared their cardiac PP2C with that of lean wild-type controls. As shown in Fig. 4B, PP2C was also increased in that obese unleptinized group. We further examined mRNA expression of cardiac PPARγ and found its levels were similar in fa/fa rats and lean littermates (1.34 ± 0.12 vs. 1.04 ± 0.28; P = 0.20) and were not altered in fa/fa rats by TGZ (1.06 ± 0.24 for TGZ-treated vs. 0.98 ± 0.4 for untreated animals).

**DISCUSSION**

Lipotoxic cardiomyopathy is a prominent component of the metabolic syndrome of leptin-unresponsive obese ZDF fa/fa rats (30). It is completely prevented by treatment with TGZ beginning at the age of 7 wk. By the age of 20 wk, untreated fa/fa rats exhibit increased myocardiag TG and ceramide, with increased DNA laddering, an index of apoptosis, and decreased contractility by echocardiogram. TGZ-treated rats, by contrast, remain virtually normal in all of the foregoing respects (30). In this report, we attempt to understand the mechanisms of the increase in lipids in unleptinized myocardium and the dramatic improvement provided by treatment with the PPARγ agonist.

Our results revealed that the defects of cardiac AMPK and PP2C are associated with lipotoxic cardiomyopathy in obese rodents. Although the mechanisms remain to be elucidated, it is possible that changes in AMPK and PP2C activity are attributable to lipid overload in the heart rather than to, for example, reduced contractility. Several lines of evidence support this idea. First, incubation of myocardium H9C2 cells with fatty acid palmitate but not with glucose leads to a decrease in P-AMPKα, suggesting palmitate or its metabolites mediates the inhibition. Previously, the same fatty acid was also shown to impair AMPK and increase ceramide synthesis and apoptosis in rat neonatal cardiac myocytes (9). In addition, metformin, an activator of AMPK (14), can override this reduction in P-AMPKα in cultured cells by fatty acids (data not shown). These results thus support cardiac lipid accumulation as a contributor to the alteration in AMPK activity. Second, cardiac AMPK activity is depressed before overt contractile dysfunction appears. In the fa/fa rat model, the earliest time point at which there was uniform evidence of heart failure in all animals was 20 wk of age (30), at least 6 wk older than the ones with reduced cardiac P-AMPKα reported here. Similarly, we found that, at <18 wk of age, ob/ob hearts show excess lipid accumulation and decreased P-AMPKα but no contractile

### Table 2. Phenotypic profiles of untreated and TGZ-treated ZDF fa/fa rats*

<table>
<thead>
<tr>
<th>Parameters (ages)</th>
<th>Untreated</th>
<th>TGZ Treated</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g (6 wk old)</td>
<td>189.3±12.2</td>
<td>191.3±7.4</td>
<td>0.7646</td>
</tr>
<tr>
<td>Body weight, g (14 wk old)</td>
<td>419.7±44.7</td>
<td>592.0±34.9</td>
<td>0.0002</td>
</tr>
<tr>
<td>Food intake, g/day (average)</td>
<td>45±6.0</td>
<td>46.8±3.0</td>
<td>0.7646</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl (14 wk old)</td>
<td>661.4±90.9</td>
<td>172.0±9.5</td>
<td>0.0002</td>
</tr>
<tr>
<td>Plasma triacylglycerol, mg/dl (14 wk old)</td>
<td>642.8±226.8</td>
<td>85.5±18.1</td>
<td>0.005</td>
</tr>
<tr>
<td>Myocardial triacylglycerol†</td>
<td>0.752±0.223</td>
<td>0.201±0.109</td>
<td>0.0028</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 6 rats in each group. TGZ, troglitazone; ZDF, Zucker diabetic fatty; FFA, free fatty acid. Data were analyzed by Student’s t-test. *Animals were untreated or treated with TGZ for 2 mo. †From Ref. 30.

---

![Fig. 3. Normalization of P-AMPKα and protein phosphatase 2C (PP2C) in ZDF fa/fa hearts by troglitazone (TGZ). ZDF fa/fa rats were treated with or without troglitazone for 2 mo, and the expression of active P-AMPKα, AMPKα, and γ-tubulin in untreated and TGZ-treated fa/fa hearts was determined by immunoblotting analysis (A). B and C: cardiac PP2C mRNA and protein expression was measured with real-time quantitative PCR and immunoblotting analysis, respectively. Bars represent means ± SE (n = 6 each) of P-AMPKα protein or PP2C normalized to γ-tubulin or 36B4, as indicated, of respective animals in each group. *P < 0.03.](http://ajpendo.physiology.org/ by 10.2203.32 on May 28, 2017)
dysfunction (data not shown; see Ref. 2). Heart failure became evident only in 24-wk-old ob/ob mice (2). In this regard, it is conceivable that the accumulation of lipids, the loss of function, and the apoptosis are likely not happening simultaneously in all cells. The lipids accumulate in a few cells initially, and those are the cells that will deteriorate first. Only when a substantial dropout of cardiomyocytes has occurred will echocardiographic evidence of failure be evident.

Our findings in fa/fa and ob/ob hearts are different from that reported in insulin-resistant JCR:LA-cp rats, which showed slightly decreased cardiac AMPK activity in spite of elevated TG in the heart (1). The reasons for the discrepancy are not clear. Notable differences are that cp rats seem to have less dyslipidemia compared with fa/fa and ob/ob animals and have no cardiac dysfunction or diabetes (1). TGZ or metformin treatment did not affect plasma lipid profiles in these rats (24). Some technical issues also make the comparison less straightforward. For instance, in their study, AMPK activity was measured with cytosolic fractions recovered from polyethylene glycol precipitation (1).

The report by Fryer et al. (7) that rosiglitazone activates AMPK in skeletal muscle suggested that a similar mechanism might prevail cardiac muscle as well. Because the obese human heart, like the heart of obese ZDF rats, has increased intramyocardial lipids (25, 26), the present findings may have clinical therapeutic implications for obese humans (25).

In fact, a reduction in active P-AMPKα was observed in untreated ZDF rats and ob/ob mice in association with an increase in PP2C, a protein phosphatase that is known to inactivate it (5, 17). Treatment with TGZ or rosiglitazone increased P-AMPKα to near-normal levels, and PP2C was reduced. Thus PP2C was increased in both models, and, in the thiazolidinedione-treated ZDF rat, it declined toward normal as P-AMPKα increased. Because the fatty acid-induced increase in PP2C has been linked to apoptosis in cultured chick neurons (11), whereas AMPK activation inhibits ceramide synthesis and apoptosis in astrocytes (3) and apoptosis in INS-1 cells (6), suppression of PP2C activity in obesity might provide a useful therapeutic target.

It is not clear from these results if the TGZ effects on the heart represent a direct action in the cardiomyocytes or are secondary to the lipopenic action of the drug. TGZ may exert its effects on PP2C and AMPK directly at the level of the heart. Treatment with TGZ in rat myocardium H9C2 cells for 24 h showed a decrease in PP2C, concurrent with an increase in P-AMPKα (data not shown), the same as that seen in the hearts of TGZ or rosiglitazone-treated obese rodents. PPARγ agonist Wy-14643, however, has no effects on PP2C and P-AMPKα levels in H9C2 cells (data not shown). Furthermore, in skeletal muscle, AMPK can also be stimulated by rosiglitazone (7). These findings are concordant with the concept that thiazolidinediones can act directly on the cardiac myocytes via PPARγ-dependent pathways. It is noteworthy that a putative PPARγ RXR response sequence (AGGTGAAAGGGA) can be identified on the rat PP2C promoter (located at −728 to −716 of transcription start site). The cardiac PPARγ pathway in obese rodents seems to be normal, since PPARγ mRNA is similar in fa/fa rats or ob/ob mice and lean controls. Alternatively, TGZ may modulate the fluxes of lipids through the heart of treated animals (10, 22) by which cardiac PP2C and AMPK activity somehow can be normalized. Stimulation of AMPK in turn would increase fatty acid oxidation (15, 16) and reduce lipid content in cardiac muscle. Further studies are needed to assess these possibilities.

In summary, our results support a role of AMPK and PP2C defects in the dysfunction of cardiac lipid metabolism in obesity and provide new insights as to the mechanisms of the liporegulatory disorder leading to lipotoxic cardiomyopathy.

ACKNOWLEDGMENTS

We thank Christie Fisher for excellent secretarial work and Daniel J. Garry, R. Haris Naseem, and Maggie Robledo for echocardiographic studies.

Table 3. Phenotypic profiles of untreated and RSG-treated ZDF fa/fa rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Untreated</th>
<th>RSG Treated</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>679.6±70.9</td>
<td>269.2±37.4</td>
<td>0.0003</td>
</tr>
<tr>
<td>Plasma triacylglycerol, mg/dl</td>
<td>485.3±203.3</td>
<td>72.7±47.3</td>
<td>0.0238</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>36.5±3.1</td>
<td>56.3±6.7</td>
<td>0.0033</td>
</tr>
<tr>
<td>Cardiac PP2C protein (arbitrary units)</td>
<td>1.00±0.49</td>
<td>0.13±0.06</td>
<td>0.026</td>
</tr>
<tr>
<td>Cardiac P-AMPKα (arbitrary units)</td>
<td>1.01±0.35</td>
<td>1.95±0.17</td>
<td>0.057</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 7 rats in each group. RSG, rosiglitazone; PP2C, protein phosphatase 2C; P-AMPKα, phosphorylated AMPKα. Data were analyzed by Student’s t-test. *Animals were untreated or treated with RSG for 2 mo. †An index of contractile function of the heart, measured by echocardiography. ‡Determined by immunoblotting analysis.
GRANTS
This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-02700 and DK-58398, Department of Veterans Affairs Merit Review, and the Jensen Diabetes Research Foundation.

REFERENCES