Reversal of chronic alterations of skeletal muscle protein kinase C from fat-fed rats by BRL-49653

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Schmitz-Peiffer, Carsten, Nicholas D. Oakes, Carol L. Browne, Edward W. Kraegen, and Trevor J. Biden. Reversal of chronic alterations of skeletal muscle protein kinase C from fat-fed rats by BRL-49653. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E915–E921, 1997.—We have recently shown that the reduction in insulin sensitivity of rats fed a high-fat diet is associated with the translocation of the novel protein kinase C, (nPKC), from cytosolic to particulate fractions in red skeletal muscle and also the downregulation of cytosolic nPKC\text{c}a. Here we have further investigated the link between insulin resistance and PKC by assessing the effects of the thiazolidinedione insulin-sensitizer BRL-49653 on PKC isoenzymes in muscle. BRL-49653 increased the recovery of nPKC isoenzymes in cytosolic fractions of red muscle from fat-fed rats, reducing their apparent activation and/or downregulation, whereas PKC in control rats was unaffected. Because BRL-49653 also improves insulin-stimulated glucose uptake in fat-fed rats and reduces muscle lipid storage, especially diglyceride content, these results strengthen the association between lipid availability, nPKC activation, and skeletal muscle insulin resistance and support the hypothesis that chronic activation of nPKC isoenzymes is involved in the generation of muscle insulin resistance in fat-fed rats.

insulin resistance; thiazolidinedione

REduced whole body response to insulin is a major feature of type II, or non-insulin-dependent diabetes mellitus, and is mainly attributable to a decrease in insulin-stimulated glucose uptake by skeletal muscle (17). Although the mechanism by which this muscle insulin resistance arises is unclear, several animal and human studies have suggested a strong link with increased tissue lipid availability (7, 23, 25, 38). For example, in the well-characterized high-fat-fed rat model of insulin resistance, which does not feature substantial hyperglycemia and hyperinsulinemia, increases in skeletal muscle triglyceride and diglyceride (DG) levels accompany the diminished capacity for insulin-stimulated glucose disposal (27).

Derivatives of triglyceride, especially DG, are activators of the protein kinase C (PKC) family of signal transduction enzymes (26). In keeping with this, we have recently shown that chronic activation and/or downregulation of specific PKC isoenzymes accompanies decreased insulin sensitivity of muscle from fat-fed rats and correlates well with muscle triglyceride and DG levels (34). Alterations in DG and PKC have also been observed in other models of insulin resistance (2, 16, 19, 21), and, because these enzymes have been shown to inhibit insulin signaling in vitro, we and others have hypothesized that chronic activation of one or more PKC isoenzymes is involved in the generation of this disorder (11, 15, 34, 36). This hypothesis should be distinguished from the controversial role of PKC in normal insulin action (5, 20). Possible targets for phosphorylation, and hence inhibition, by PKCs include early components of the insulin signal transduction pathway, especially the adaptor molecule insulin receptor substrate-1 (IRS-1) (14), as well as metabolic enzymes such as glycogen synthase (1).

The PKC family can be divided into three groups on structural and functional bases (see Ref. 26 for review): the conventional PKC isoenzymes α, β, and γ, which are dependent on calcium, DG, and phospholipid for activity; the novel (n)PKC isoenzymes δ, ε, ζ, and η, which are calcium independent; and the atypical PKC isoenzymes ι and ι, which are independent of both calcium and DG. Activation of PKC in the presence of these lipids occurs at cell membranes, and hence a reduction in cytosolic PKC and its recovery in membrane fractions are frequently taken as a measure of activation of the kinase (26). Consequently, the decreased proportion of nPKC observed in the cytosolic fraction of muscles from fat-fed rats, relative to starch-fed controls (34), suggests that activation of this isoenzyme accompanies the reduction in insulin sensitivity. Furthermore, chronic activation of PKC isoenzymes can lead to their proteolysis (12, 41). Thus in the red muscle of fat-fed rats there is a reduction in the total levels of nPKC\text{c}a, apparent only in the cytosolic component (34), which is suggestive of the downregulation of this kinase, possibly after prolonged stimulation.

The current studies were undertaken to test whether the changes in cytosolic nPKC levels in muscles from fat-fed rats would be reversed by the insulin-sensitizing compound BRL-49653, which reduces systemic lipid supply and utilization (28). This prediction follows from the hypothesis that chronic alterations in lipid activators and hence PKC activity in muscle lead to a reduction in insulin sensitivity. Our results confirm that manipulation of insulin sensitivity, by fat feeding and drug treatment, is associated with alterations in the levels and distribution of specific nPKC isoforms.

MATERIALS AND METHODS

Materials. Rabbit antipeptide antibodies against PKCs α, β, δ, ε, and ζ were from GIBCO BRL, Life Technologies, Mulgrave, Australia. Rabbit antipeptide antibody against nPKC\text{c} was from Santa Cruz Biotechnology, Santa Cruz, CA. Horseradish peroxidase-linked donkey anti-rabbit antibody was from Jackson Immuno Research Laboratories, West Grove, PA. Renaissance-enhanced chemiluminescence reagents were from NEN, Boston, MA. Other biochemicals were mostly from Sigma Chemical or BDH Laboratory Supplies.

Experimental animals and dietary treatment. All experimental procedures performed for this study were approved by
the Animal Experimentation Ethics Committee (Garvan Institute) and were in accordance with the National Health and Medical Research Council of Australia guidelines on animal experimentation. Procedures were carried out as described previously (28). Briefly, male Wistar rats weighing ∼250 g were fed isocaloric diets (350 kJ/day) of either a high-starch or a high-fat diet for 3 wk up to the study day. The composition of the fat diet was 59% fat, 21% protein, and 20% carbohydrate (38); the starch diet consisted of 10% fat, 21% protein, and 69% carbohydrate (23). Starch- and fat-fed rats were divided into BRL-49653-treated and control subgroups. Treated rats were given four doses of BRL-49653 (10 mmol·kg\(^{-1}\)·day\(^{-1}\)) at 0730 by gastric gavage, commencing 3 days before and finishing on the day of killing. Control rats were gavaged with an equal volume of vehicle (saline). Body weights of rats after 3 wk of diet feeding were 347.5 ± 5.48 (starch-fed, untreated), 348.2 ± 1.5 (starch-fed, treated), 378.5 ± 4.7 (fat-fed, untreated), and 376.2 ± 4.2 (fat-fed, treated), n = 6 in each group.

Tissue extraction and immunoblotting. Red gastrocnemius and red quadriceps muscles were collected rapidly after animals were killed by pentobarbitone overdose, frozen with liquid \(N_2\)-cooled tongs, and stored at \(-80^\circ\)C. Muscle cytosolic and solubilized-particulate fractions were prepared as described previously (34) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (~50 and 25 mg protein, respectively). Proteins were electrophoresed onto nitrocellulose membranes, which were probed with rabbit antipeptide antibodies specific for PKC isoenzymes α, δ, ε, Η, or ζ, followed by horseradish peroxidase-linked donkey anti-rabbit antibody (34). PKC isoenzymes were then visualized by incubation of the membrane with enhanced chemiluminescence reagents and exposure to X-ray film for between 30 s and 10 min. Densitometry of PKC bands was carried out using a Medical Dynamics Personal Densitometer SI and analyzed using IP Lab Gel H software (Signal Analytics, Vienna, VA). The relationship between the amount of sample subjected to immunoblotting and the signal intensity observed was linear under the conditions described above. As previously, the ratio of the dry weight of the detergent-insoluble fraction to the wet weight of the starting material was used to correct for variations in recovery between samples (34). This measurement exhibited low variability and was not significantly different between starch- and fat-fed rats [e.g., 5.48 ± 0.08 (n = 6) vs. 5.41 ± 0.04 (n = 6), respectively, in one experiment].

Presentation of results. To study the effects of BRL-49653 on PKC, four groups of rats were examined: starch-fed untreated, starch-fed drug-treated, high-fat-fed untreated, and high-fat-fed drug-treated. The mean cytosolic PKC content of starch-fed untreated rat muscle was set to 100% in each case, and the mean cytosolic PKC of muscle in the other groups was expressed relative to this. This is the optimal measurement, since any change in either the location or total content of a specific nPKC in the muscles was clearly reflected in the cytosolic component. Indeed, isoenzymes that exhibited downregulation did so only in cytosolic fractions, whereas membrane-associated levels remained unchanged (34). Furthermore, cytosolic PKC measurements showed less variability between samples from the same group than those of membrane-associated PKC, most likely because they were obtained after fewer experimental procedures that could affect recovery. For the estimation of total PKC content, cytosolic and membrane amounts were added. We have previously determined that only traces of PKC isoenzymes are detected in the detergent-insoluble fraction (34), and these were not quantitated.

Statistics. All results are expressed as means ± SE. Analysis of diet and treatment effects was by unpaired Student's t-test. Statistical calculations were performed using Statview SE + GraphicsTM for Macintosh (Abacus Concepts, Berkeley, CA).

RESULTS

PKC\(_{α}\) and atypical PKC\(_{ζ}\) are not affected by fat feeding in skeletal muscle (34), but we included measurements of these isoenzymes in the study to assess more fully the effects of BRL-49653 on the PKC family. No significant effects of diet or BRL-49653 treatment were observed on either PKC\(_{α}\) (Fig. 1A, diet effect \(P < 0.25\), drug effect \(P > 0.75\), both muscle types combined) or PKC\(_{ζ}\) (Fig. 1B, diet effect \(P > 0.70\), drug effect \(P > 0.65\), both muscle types combined), which therefore
serve as useful negative controls when considering changes in other PKC isoenzymes.

There was a fall in cytosolic levels of nPKC\(_e\) in both red quadriceps and red gastrocnemius muscles of untreated rats in response to fat feeding (Fig. 2A), confirming previous results (34). Although total amounts did not change (Fig. 2B), there was also a corresponding rise in membrane-associated levels, consistent with a translocation of this isoenzyme (Fig. 2C). Treatment with BRL-49653 did not significantly affect cytosolic nPKC\(_e\) in either muscle from starch-fed rats but was able to partly reverse the fall in cytosolic levels seen in muscle from fat-fed rats (Fig. 2A, \(P < 0.01\) for fat-fed treated vs. fat-fed untreated, both muscle types combined). As a consequence, the ratio of membrane-associated to cytosolic nPKC\(_e\) in muscle from these rats fell by over 30% (Fig. 2C), so that the alteration due to fat feeding was overcome by \(\sim 50\%\).

Fat feeding also caused a decrease in the amount of cytosolic nPKC\(_e\) in both muscles (Fig. 3A), but this was not accompanied by changes in membrane-associated kinase, which meant that total levels of this isoenzyme were diminished in muscles from fat-fed rats (Fig. 3B), whereas the ratio of membrane-associated to cytosolic protein increased (Fig. 3C). When rats were treated with BRL-49653, no change was again observed in the cytosolic levels of nPKC\(_e\) in muscles from starch-fed rats (Fig. 3A). However, the decrease seen in muscles from fat-fed rats was partially reversed (Fig. 3A; \(P < 0.002\) for fat-fed treated vs. fat-fed untreated, both muscle types combined). This was also apparent in the total levels of nPKC\(_e\) in muscle from fat-fed rats, which were increased by 55% on treatment with the drug (Fig. 3B). The ratio of membrane-associated to cytosolic nPKC\(_e\) therefore also fell (Fig. 3C).

Similar results were also seen in the case of nPKC\(_u\) (Fig. 4), in that cytosolic and hence total levels of this kinase fell in muscles from fat-fed rats, although the changes were less pronounced than those exhibited by nPKC\(_u\). Treatment with BRL-49653 tended to elevate cytosolic nPKC\(_u\) in fat-fed rats, which was best demonstrated as a decrease in the ratio of membrane to cytosolic levels of the isoenzyme (Fig. 4C).

**DISCUSSION**

This study was designed to test further the hypothesis that chronic activation of PKC isoenzymes in red skeletal muscle is associated with diminished insulin sensitivity, since this would help to provide a mechanistic explanation of the link between increased availability of lipid and the generation of insulin resistance. By employing a high-fat diet, previously shown to reduce red skeletal muscle insulin sensitivity by nearly 50% (38), we have been able to demonstrate chronic changes in cytosolic nPKC levels. Most importantly, use of BRL-49653, previously found to improve muscle insulin sensitivity in high-fat-fed and Zucker fa/fa rats (9, 28), partially reverses the defined chronic alterations in nPKCs. Because the drug
also lowers plasma free fatty acid levels (28) and the DG content of muscle from fat-fed rats (27), the present work strengthens the argument for a role of PKC isoenzymes in mediating the decreased insulin sensitivity elicited by increased lipid availability, on stimulation of the kinases by lipid activators. Although we have previously shown that assays of PKC activity are broadly consistent with the results

Fig. 3. Effect of high-fat feeding and treatment with BRL-49653 on nPKC\textsubscript{u}. Results obtained using nPKC\textsubscript{u}-specific antibodies are expressed as in Fig. 2. Significance of contrast: fat-fed untreated vs. starch-fed untreated *\(P < 0.05\), **\(P < 0.02\), †\(P < 0.002\); fat-fed treated vs. fat-fed untreated ‡\(P < 0.05\).

Fig. 4. Effect of high-fat feeding and treatment with BRL-49653 on nPKC\textsubscript{d}. Results obtained using nPKC\textsubscript{d}-specific antibodies are expressed as in Fig. 2. Significance of contrasts: fat-fed untreated vs. starch-fed untreated *\(P < 0.05\); fat-fed treated vs. fat-fed untreated ‡\(P < 0.02\).
obtained by immunoblotting (34), the assays are optimal for conventional PKC rather than nPKC activity and are subject to artifacts, as discussed (34). In addition, PKC assays are less informative than immunoblotting, which reveals the behavior of individual isoenzymes, and we have therefore restricted the PKC measurements in the present study to the latter. Our results suggest that these nPKC isoenzymes could mediate inhibitory effects on insulin signaling. nPKC_\alpha_2 appears to be chronically activated without showing signs of downregulation, possibly in response to increased DG levels in muscle. In contrast, the changes in nPKC_\alpha_ and nPKC_\gamma_ were suggestive of both translocation and downregulation, although the decrease in cytosolic nPKC_\gamma_ was less marked than that in nPKC_\alpha_. This, together with our previous observation that fat feeding also alters nPKC_\gamma_ in white skeletal muscle that is relatively insensitive to insulin (34), suggests that changes in this isoenzyme may be less directly related to insulin sensitivity.

Our observations of the cytosolic depletion of nPKC_\gamma_ could be interpreted as an indication that in fat-fed rats, nPKC_\gamma_ is constantly translocating to the membrane fraction, where it is subsequently downregulated by proteolysis. A greater turnover of nPKC_\gamma_ at the membrane might lead to increased phosphorylation of protein substrates and thus inhibition of insulin signaling, even though the rate of nPKC_\gamma_ synthesis can no longer maintain cytosolic nPKC_\gamma_ at those levels seen in the starch-fed control rats. Although the physiological functions of nPKC_\gamma_ have not been determined, it is interesting to note that this isoenzyme is poorly expressed in most tissues but is a major PKC in skeletal muscle (29).

Potential targets for PKC in disrupting insulin signaling have mainly been identified in vitro and include the inhibition of the insulin receptor tyrosine kinase by phosphorylation of the receptor β-subunit (6), inhibition of insulin-stimulated phosphorylation of IRS-1 (14), inhibition of phosphatidylinositol 3-kinase activation (13) and stimulation of tyrosine phosphatase activity (35). In addition PKC may directly phosphorylate and inhibit glycogen synthase (1). However, the precise mechanisms by which PKC isoenzymes act in the generation of insulin resistance remain to be determined.

In some previous studies, acute insulin stimulation of tissues or cells has led to translocation of PKC isoenzymes from soluble to membrane fractions (4, 37, 40). Although the role of PKC in normal insulin signaling is also uncertain (5, 20), recent evidence suggests that atypical PKCs such as PKC_\gamma_ could be involved in the insulin-mediated stimulation of p21^{ras} (18), gene transcription (39), and glucose transport (4), whereas the translocation of DG-sensitive isoenzymes could have inhibitory effects on glycogen synthase activity (4). Our data are consistent with these possibilities, as we observe translocation of DG-sensitive PKC isoenzymes but not of atypical isoenzymes in insulin-resistant muscles. We have previously described an acute redistribution of PKC_\gamma_ in skeletal muscle during euglycemic-hyperinsulinemic clamp (34). Because this translocation was greater in fat-fed rats, compared with starch-fed controls, it may not be a proximal signal but instead a secondary effect due to insulin-stimulated changes in lipid flux. As glucose uptake into the muscles from fat-fed rats was reduced (34), this again argues for an inhibitory effect of PKC_\gamma_ on insulin signaling. Acute effects of insulin on lipids and PKC isoenzymes would make the interpretation of the effects of BRL-49653 treatment more complicated, and in the present study we have concentrated on chronic PKC alterations in basal fat-fed rats.

A recent study of PKC isoenzyme expression in tissues of the type II diabetic Goto-Kakizaki rat (2) found more widespread changes in skeletal muscle PKC, relative to nondiabetic controls, than those seen here. Translocation of PKC_\alpha_2, PKC_\beta_\iota, and PKC_\gamma_ were observed in addition to that of nPKC_\gamma_, whereas nPKC_\xi_ levels were again depleted. It was suggested that hyperinsulinemia played a role in the elevation of DG and activation of PKC, because similar changes in PKC isoenzymes were observed in normoglycemic, hyperinsulinemic obese aged and obese Zucker rats (2). However, gross hyperinsulinemia is not evident in the high-fat-fed rat (34), and we suggest that the more specific changes observed here in nPKCs arise from increased lipid availability to skeletal muscle. Consistent with that interpretation, it should also be noted that BRL-49653 treatment reduces plasma insulin levels in both starch- and fat-fed rats (28) but affects muscle PKC, along with DG and insulin sensitivity, in fat-fed rats alone (Fig. 1, B-D, and Refs. 27 and 28).

Another thiazolidinedione, troglitazone, has also been demonstrated to affect membrane-associated nPKC_\gamma_ and nPKC_\xi_ of ventricular cardiomyocytes (3). However, in that study, a 30-min incubation with the drug did not reduce translocation of PKC protein in response to the DG analog phorbol 12-myristate 13-acetate (PMA) but indirectly caused partial inhibition of the PKC activity that could be measured in membrane fractions after PMA treatment. This acute effect of troglitazone was accompanied by improvement of PMA-induced desensitization of insulin-stimulated glucose transport (3), in agreement with our findings that activated PKC is associated with insulin resistance. Because acute treatment with troglitazone did not affect PKC distribution (3), this argues against a direct effect of thiazolidinediones on the kinases in muscle from fat-fed rats. Although we did observe reduced translocation of nPKC isoenzymes in fat-fed rats treated with BRL-49653, this difference may be explained by the chronic nature of the treatment in our study (over 4 days) and suggests different mechanisms both for the generation of insulin resistance by PKC, activated either acutely by PMA or chronically by fat-feeding, and for its amelioration in the shorter and longer term.

Thiazolidinediones have been found to improve several aspects of insulin-mediated glucose metabolism, including glucose disposal rate, in a large number of studies (see Ref. 33 for review). To date, the only molecular target that has been identified for thiazolidinediones is the peroxisome proliferator-activated receptor γ, which is substantially expressed only in...
Indeed, downregulation of cytosolic nPKC isoenzyme may be less sensitive to such activation. Lipids, may also help to explain why, in contrast to our and the elevated DG. Cellular compartmentalization, possible separate cellular localization of the isoenzyme was unaffected (22). However, as evidence for regulation of different DG species in distinct pools in skeletal muscle has also been reported (10), an alternative explanation for the lack of diet effect on PKC, is a possible separate cellular localization of the isoenzyme and the elevated DG. Cellular compartmentalization, as well as stimulation of nPKCs by as yet undefined lipids, may also help to explain why, in contrast to our findings with BRL-49653 in the fat-fed rat (27), muscle DG was in fact elevated by pioglitazone in the KKA\textsuperscript{y} mouse (31), whereas insulin action was improved in each case.

In conclusion, the data presented here are consistent with one or more PKC isoenzymes playing a causative role in the generation of fat-induced insulin resistance. Specific alterations in nPKCs are observed in response to high-fat feeding, previously shown to cause a reduction in skeletal muscle insulin sensitivity, and are partially reversed by treatment with an antidiabetic agent. Interpretation of these observations is not complicated by the presence of hyperglycemia or hyperinsulinemia in the fat-fed rats but again suggests that increased lipid availability is an important factor. To determine whether the affected nPKC isoenzymes do attenuate insulin signaling it will be necessary to identify substrates in muscle for these kinases, which might affect insulin action, and demonstrate that these undergo changes in phosphorylation state and activity in insulin-resistant states.

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