Endothelial dysfunction in Type 2 diabetes correlates with deregulated expression of the tail-anchored membrane protein SLMAP

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Submitted 12 January 2005; accepted in final form 9 March 2005

TYPE 2 DIABETES leads to a marked increase in cardiovascular complications, and about 75% of patients die from microvascular and macrovascular disease (18, 21). The cardiovascular complications and specifically the linkage to endothelial dysfunction that are seen at an early stage in patients with diabetes are considered as the major risk factor for both Type 1 and Type 2 diabetes (9, 12, 13, 29, 37–39). The etiology of Type 2 diabetes is complex and involves both genetic predisposition as well as environmental factors, notably, lifestyle and dietary influences. A number of studies have also suggested that hyperglycemia may have long-lasting effects on vascular function resulting from changes in gene expression that are not readily reversible despite the correction of metabolic dysfunction (13, 34). In this regard, a great deal of effort is being directed to defining genes that may be differentially expressed in diabetics as this could provide an understanding of the disease process as well as lead to the discovery of important targets for therapeutic intervention. It is envisioned that the identity of genes whose expression is specifically altered in Type 2 diabetes will prove to be of great importance for the therapeutic intervention of diabetes-related disorders.

Mouse models of Type 2 diabetes have become an important tool for determining the molecular basis for disease progression and tissue dysfunction. db/db Mice provide a monogenic animal model of Type 2 diabetes that lack functional leptin receptors and thus are hyperphagic and massively obese with marked hyperglycemia (11). Our recent studies and those of others indicate that altered vascular and cardiac function is prevalent in the db/db mouse (1–5, 10, 22, 28–31, 36). Thus this model may be well suited to investigate the cellular and molecular basis of cardiovascular disease progression in Type 2 diabetes as well as drug discovery. In this regard, peroxisome proliferator-activated receptor (PPAR)-γ ligands reduce insulin resistance and therefore are utilized in the pharmacological treatment of Type 2 diabetes (7, 8, 20). Carley et al. (10) have reported that db/db mice treated with the novel nonthiazolidinedione PPAR-γ agonist and insulin sensitizing agent 2-[2-(4-phenoxy-2-propylphenoxy)ethyl]indole-5-acetic acid (COOH) had reduced blood glucose levels and enhanced insulin-stimulated glucose uptake in cardiomyocytes. Although COOH-treated db/db mice demonstrated beneficial metabolic changes, COOH treatment did not improve contractile performance as assessed with ex vivo perfused working hearts and in vivo by echocardiography. PPAR-γ is a member of the nuclear receptor superfamily and thus functions as a ligand-activated transcription factor to regulate gene expression (7, 8, 33). Recent data suggest that db/db mice also experience vascular dysfunction characterized by defects in endothelial/smooth muscle cell signaling. We report here that endothelial dysfunction in db/db mice is associated with a significant upregulation of the expression of the tail-anchored membrane protein sarcolemmal membrane-associated protein (SLMAP), which is directed to cellular membranes by a carboxy-terminal anchor and has been...
shown to be a component of the excitation-contraction coupling apparatus in muscle cells (17, 40, 41). Furthermore, COOH treatment can reverse the endothelial dysfunction in db/db mice and correct the aberrant expression of SLAP in vascular tissue. Although the exact function of SLAP remains elusive, it has been shown to be a component of the centrosome implicated in the regulation of cell’s mitotic activity as well as membrane function and signal transduction (15, 16, 17). The observations here suggest that SLAP expression is modulated by PPAR-γ-related mechanisms and may be an important indicator of microvascular function at various levels including subcellular membrane biology and mitotic activity.

**METHODS**

**Animals and tissue handling.** Eight- to sixteen-week-old male C57BL/Ks-lepr+/lepr- (db/db) mice and nondiabetic lean heterozygote (db/+ ) controls were purchased from Jackson Laboratories (Bar Harbor, ME). In accordance with a protocol approved by the University of Calgary Animal Care Committee, mice were killed by cervical dislocation. The heart and mesenteric arcade were dissected out in physiological-buffered saline solution for Western blot analysis and real-time PCR and Krebs solution for functional studies.

**Drug intervention protocol with COOH.** At 8 wk of age, each type of animal was divided into three groups: group 1 received 30 mg·kg-1·day-1 COOH [gift of Merck Pharmaceuticals; see Carley et. al. (10) regarding the dosage] in powder chow for 8 wk; group 2 received regular powdered chow for 8 wk; and group 3 received 30 mg·kg-1·day-1 COOH for 8 wk and were then crossed over to untreated feed for a further 5 wk (this we have termed the “crossover” study).

**Experimental protocols.** Vascular smooth muscle reactivity to phenylephrine (PE) and endothelium-dependent relaxation to acetylcholine (ACh) were studied using a Mulvany-Halpern myograph as previously described (27, 28, 29, 30, 31). After a 45-min equilibration period, the vascular reactivity to PE was studied in second-order small mesenteric arteries (SMAs) from db/+ and db/db mice. Maximum contraction was expressed as milliNewtons per millimeter of SMA. After 30 min of stabilization, endothelium-dependent vascular relaxation to ACh was recorded in preparations contracted with a submaximal concentration of PE (EC75–80).

**Real-time PCR.** Total RNA was extracted from mesenteric arteries using an RNeasy Mini Kit with on-column DNase treatment (Qiagen), and reverse PCR primers were designed (15, 16, 17). The observations here suggest that SLAP activity as well as membrane function and signal transduction may be an important indicator of microvascular function at various levels including subcellular membrane biology and mitotic activity.

**Results**

**Vascular dysfunction in db/db mice.** The data from the present study demonstrate that 8-wk treatment of db/db mice with 30 mg·kg-1·day-1 COOH resulted in a reduction in blood glucose as well as triglycerides (P < 0.05) but no change in total cholesterol. Conversely, the body weights of control nondiabetic (db+) mice were unaffected by treatment with COOH and nor were triglycerides; however, cholesterol was lowered (Table 1). In the crossover component of the study, the formerly COOH-treated db/db mice reverted to a hyperglycemic state.

**Table 1. Metabolic parameters**

<table>
<thead>
<tr>
<th></th>
<th>Body Weight, g</th>
<th>Blood Glucose, mM</th>
<th>Triglycerides, mM</th>
<th>Cholesterol, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>db/+ Mice</td>
<td>30±1.4</td>
<td>12.0±0.8</td>
<td>1.2±0.1</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>db/+ Mice after treatment</td>
<td>32±1.3</td>
<td>12.0±1.0</td>
<td>0.94±0.1</td>
<td>1.35±0.1*</td>
</tr>
<tr>
<td>db/db Mice</td>
<td>51.5±1.6</td>
<td>48±2.5</td>
<td>2.1±0.6</td>
<td>2.8±0.1</td>
</tr>
<tr>
<td>db/db Mice after treatment</td>
<td>54±1.8</td>
<td>13±0.5*</td>
<td>1.0±0.2*</td>
<td>2.7±0.15</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data for body weight, blood glucose, triglycerides, and cholesterol before and after 2-[2-phenoxyl-2-propylphenoxy]ethylindole-5-acetic acid in db/+ and db/db mice are shown. *P < 0.05.
SLMAP EXPRESSION IN DIABETES

Fig. 1. Vascular reactivity in diabetic (db/db) mice. A: effect of phenylephrine (PE) in small mesenteric arteries (SMAs) from db/db mice. Concentration-dependent contractile effects of PE on SMAs from control (db/+ ) and db/db mice are shown (n = 6 for each group, *P < 0.05). B: mean concentration-relaxation response curves to ACh in SMAs from db/+ (n = 6) and db/db (n = 7) mice (*P < 0.05). Points are mean values with SEs mean shown by vertical bars.

Maximum contraction and sensitivity expressed as pEC50 values were 3.0 ± 0.4 mN/mm and 6.5 ± 0.1 for db/+ mice and 4.3 ± 0.3 mN/mm and 6.5 ± 0.1 for db/db mice, respectively. Maximum contraction to PE was significantly enhanced (P < 0.01) without a change in the sensitivity in mesenteric arteries from db/db mice compared with db/+ controls.

ACh-induced maximum relaxation of SMAs was significantly reduced (P < 0.01) in db/db compared with db/+ mice (Fig. 1B). Sensitivity (pEC50) and maximum relaxation to ACh were 6.9 ± 0.1 and 92 ± 2% for db/+ mice and 6.7 ± 0.2 and 42 ± 1% for db/db mice, respectively. Thus the contractile and relaxation properties of SMAs were markedly altered in mice with Type 2 diabetes.

Expression of SLMAP protein in the db/db mouse. Specific antibodies raised against SLMAP fusion protein were used to study SLMAP expression in the db/db mouse heart and mesenteric arteries. Cardiac and mesenteric tissue were harvested and analyzed by Western blot analysis. Figure 2B shows that anti-SLMAP recognized three polypeptides of ~80, 41, and 35 kDa in hearts from both db/+ and db/db mice (lanes 1 and 2, respectively). The 35-kDa polypeptide was the most abundantly expressed in cardiac muscle from the mouse heart. This profile of expression of SLMAP isoforms is consistent with that reported previously for the rabbit myocardium (41). Interestingly, there was no change in the expression of any of the SLMAP isoforms in cardiac tissue from db/+ versus db/db mice.

The SLMAP expression profile in the mesenteric vessels revealed the presence of a predominant immunoreactive polypeptide of 41 kDa and the 35-kDa SLMAP isoform (Fig. 2A). It is notable that the level of expression of the 35-kDa SLMAP protein in mesenteric arteries from db/db mice was markedly higher than that in the vasculature of db/+ mice (Fig. 2B, lanes 1 and 2). The 80-kDa isoform was not detected in mesenteric arteries from db/+ or db/db mice under these assay conditions. The protein loading for db/+ versus db/db mice was identical as shown by the level of actin expression. The Western blot data showing the SLMAP expression from the db/db myocardium and mesenteric vessels were quantified using densitometry, and it was evident that db/db mice exhibit a ~2.7-fold increase in expression of the 35-kDa SLMAP isoform in the vasculature, whereas the expression of this isoform remained unaltered in cardiac muscle (Fig. 2C). There was no change in the expression of the 41-kDa isoform in SMAs (Fig. 2C). Thus these results imply that it is the expression of the mesenteric 35-kDa SLMAP isoform that is specifically enhanced in db/db mice.

COOH can restore vascular function and depress SLMAP mRNA expression. The effects of COOH on vascular function and SMLAP expression in SMAs from db/db mice were examined. Figure 3A shows the vascular defect in db/db SMAs in the form of a depressed response to ACh. The reduced relaxation to ACh in db/db mice was entirely reversed by prior treatment of the animals with COOH. Interestingly, the elevated vascular reactivity to PE observed in db/db mice (Fig.

Fig. 2. Expression of sarcolemmal membrane-associated protein (SLMAP) isoforms in vasculature and cardiac tissue in db/db mice. SMA (A) or heart (B) tissue was isolated and analyzed by immunoblotting with anti-SLMAP. Anti-SLMAP-immunoreactive proteins are depicted by arrows, and the positions of migration of the molecular weight standards are shown as numbers (in kDa). Actin expression was monitored as a control for loading. C: expressions of 41- and 35-kDa SLMAP from SMAs and hearts of both db/+ and db/db mice were quantified by densitometry and are shown as a bar graph. *P < 0.05.
A) was not reversed after COOH treatment (data not shown). Furthermore, during a crossover study where COOH was omitted from the diet of db/db mice, the vascular dysfunction in terms of the relaxation response to Ach was seen to return to pretreatment levels (Fig. 3A).

To further investigate changes in SLMAP in mesenteric arteries and whether there was a correlation with endothelial and/or vascular contractile dysfunction, we performed real-time PCR on RNA samples from db/+ and db/db mice and compared this with SLMAP transcript levels in the vasculature from diabetic mice in which the endothelial dysfunction, but not vascular contractile dysfunction, was reversed by COOH treatment. In addition, mRNA levels of SLMAP were much higher in the vessels of untreated animals compared with COOH-treated animals (Fig. 3B). Compared with the housekeeping gene β-actin, the untreated group had 5.7 × 10⁻²-fold relative expression of SLMAP, whereas it was nondetectable in the drug-treated group or the db/+ group. When therapy was discontinued in the crossover study, the SLMAP levels were seen to return to 6.3 × 10⁻⁴-fold relative to β-actin. The threshold cycle for β-actin for all groups was consistent and comparable, ensuring that the results were not a function of variations in the amount of starting material. These data reveal a specific correlation between endothelial dysfunction and SLMAP expression levels in SMAs of db/db mice.

**DISCUSSION**

The data from the present study reveal that in the microvasculature of the db/db mouse there is an upregulation of the 35-kDa isoform of the tail-anchored membrane protein SLMAP that correlates with endothelial dysfunction. Treatment of the db/db mouse with the PPAR-γ agonist COOH reversed not only endothelial dysfunction but also reduced the expression of SLMAP.

SLMAP isoforms of 35 and 41 kDa were expressed in both vascular and cardiac tissue from db/db and control mice, although the level of expression of various isoforms varies between these tissues. The expression of the 35-kDa SLMAP protein and SLMAP mRNA in vascular, but not cardiac, tissue was upregulated in the db/db mouse. Treatment with the PPAR-γ agonist COOH corrected both plasma glucose and triglyceride levels but specifically affected SLMAP expression in vascular tissue. Furthermore, COOH treatment normalized endothelial function in vascular tissue but did not improve either vascular reactivity or, as previously reported, cardiac function (10). These data are suggestive that abnormalities in the regulation of SLMAP expression in the db/db mouse may be linked to endothelial dysfunction. A single gene that encodes SLMAP maps to the human chromosome 3p14.2–21 region (40, 41), a region that also has been reported to carry genes linked to Type 2 diabetes-related disorders (26). Whether the SLMAP gene locus is involved in these diabetic patients now needs to be further investigated.

The cellular and molecular bases for cardiovascular dysfunction in Type 2 diabetes remain to be defined; however, there is increasing evidence of a prognostic link between endothelial dysfunction and the later development of both micro- and macrovascular complications in diabetic populations (24, 38, 39). The selective impairment of agonist-stimulated endothelium-dependent relaxation is seen as a very early stage in the development of vascular disease and, in the SMA of the db/db mouse, has been associated with a reduction in the bioavailability of endothelial cell-derived nitric oxide (NO), which can be reversed by the dietary provision of sepiapterin [a source of the important cofactor for endothelial NO synthase (eNOS), tetrahydrobiopterin] (28, 30). No change, however, was reported in the sensitivity to an endothelium-independent vasodilator, sodium nitroprusside (22, 28). Furthermore, no change in the expression of eNOS RNA or protein but enhanced intracellular oxidative stress has also been reported in the SMA from the db/db mouse (31). Comparable changes have been reported for the coronary arterioles from the db/db mouse (2). Collectively, these data indicate that the loss of eNOS-derived NO that results from an increase in oxidative stress leads to endothelial dysfunction; however, the cellular processes that link these events remain unclear.

The data presented in the present study agree with previous studies that have shown that leptin receptor-deficient (db/db) mice exhibit higher body weight compared with nondiabetic littermates (db/+), and typical features of Type 2 diabetes with...
hyperglycemia and hyperinsulinemia (1, 2, 4, 5, 10, 35, 36). The studies we report show that the functional properties of the vasculature in the db/db mouse are significantly impaired in terms of contraction and endothelium-dependent relaxation. These data are in agreement with our previous findings with SMAs from db/db mice (28, 30, 31) and comparable to the endothelial dysfunction reported for coronary arteries from the same mouse model of Type 2 diabetes (2, 3). This functional impairment was closely associated with a marked dysregulation in SLMAP in the db/db microvasculature. The data reveal that the contractile response to PE in the db/db vasculature was significantly increased, whereas the relaxation induced by ACh was depressed, consistent with previous results (28, 30). We (28) and Lagaud et al. (22) have previously reported that endothelium-independent relaxation to sodium nitroprusside was not altered in the db/db mouse, thus suggesting that the cellular mechanisms in the vascular smooth muscle cells mediating vasodilatation are not altered in the diabetic state, and similar conclusions were reached by Bagi et al. (2). Furthermore, the enhanced reactivity of vascular smooth muscle to vasoconstrictor stimuli in the db/db mouse has been linked to changes in the cyclooxygenase-mediated pathway (22, 31). In the present study, the alteration in endothelial function in the db/db vasculature was accompanied by a 2.7-fold increase in the expression of the 35-kDa SLMAP isoform. It is notable that SLMAP upregulation was specific to the vascular tissue as it was unaltered in the myocardium from these animals. These data thus reinforce our conclusion that the deregulation of SLMAP expression in the vasculature is linked to endothelial dysfunction.

COOH is a selective nonthiazolidinedione PPAR-γ agonist (10), which, like thiazolidinediones (glitazones), possesses insulin sensitizing activity (19). Carley et al. (10) reported that chronic oral administration of COOH to db/db mice resulted in normoglycaemia and enhanced insulin-stimulated glucose uptake into isolated cardiomyocytes but did not improve contrac tile function as assessed with ex vivo perfused heart and in vivo electrocardiography studies. In the present study, SLMAP expression was neither altered in the cardiac tissue from db/db mice nor was it affected by treatment with COOH. These data indicate that the effects of COOH on SLMAP expression may be selective for the vasculature.

PPAR-γ ligands have a variety of effects on vascular tissues, such as inhibition of atherogenesis (7, 8, 19). These vascular effects could be mediated directly by activation of PPAR-γ expressed in vascular tissues and/or by indirect mechanisms secondary to improved diabetic status due to insulin sensitization. The intervention with COOH corrected endothelial dysfunction, as assessed by the measurement of endothelium-dependent relaxation to ACh and normalized metabolic abnormalities but not vascular reactivity to PE. These changes were accompanied by a complete reversal and downregulation of SLMAP mRNA as assayed by real-time PCR. Furthermore, after “crossover” to a 5-wk period “off” COOH therapy, vascular dysfunction was seen to return, accompanied by a significant upregulation in SLMAP mRNA levels. Activated PPAR-γ serves to regulate the activity of various genes involved in insulin resistance, and our data here demonstrate that the repression of the SLMAP gene may also be under the regulation of this nuclear receptor. These data suggest that the prevention of endothelial dysfunction with COOH treatment may be secondary to the improvement of the metabolic profile of db/db animals. Furthermore, because treatment with COOH corrected the hyperglycemia and elevated plasma triglycerides as well as SLMAP expression in the vasculature, it is conceivable that SLMAP expression in the vasculature may also be regulated by metabolic factors. This conclusion may, however, be premature as Bagi et al. (2) have reported that a 1-wk treatment of db/db mice with rosiglitazone reversed endothelial dysfunction in the coronary arteries of db/db mice but without any significant action on the metabolic abnormalities. Further studies are therefore required to better understand the regulation of the tissue-specific expression of the SLMAP gene and its regulation by PPAR-γ activation.

The data presented here suggest a close link between the dysregulation of SLMAP expression and changes in endothelial function of the SMA of the db/db mouse. The vascular myopathy in these diabetic animals may reflect alterations in calcium signaling because excitation-contraction coupling and vasoconstrictor and eNOS-dependent responses are all mediated by calcium (6, 32). Recent studies show that SLMAPs are components of cell surface membranes, caveolae, and the sarcoplasmic reticulum in cardiac and muscle cells and may serve roles in excitation-contraction coupling mechanisms (16, 17). Altered calcium handling may also be a common feature of diabetes (5, 32). Thus, although the specific role of SLMAP in the regulation of endothelial function remains to be determined, it is well established that caveolae are an essential component of the regulation of eNOS in endothelial cells (25), and a reduction in the bioavailability of endothelium-derived NO is a common feature observed in vascular disease (12, 13, 37). Changes in caveolae function are also a likely important contributor to vascular disease (14). Thus it is quite conceivable that SLMAPs may also regulate caveolae-endoplasmic/sarcoplasmic reticulum function and calcium signaling in vascular tissue, and this may directly or indirectly affect the cellular mechanisms involved in the regulation of endothelial function. Altered levels of SLMAP at these sites may lead to deregulation of signal transduction mechanisms and the microvascular dysfunction observed in the db/db vasculature. In addition, the upregulation of SLMAP may also effect cell growth and centrosome function (15), which may lead to changes in endothelial cell dysfunction in diabetic states (13).

In conclusion, vascular SLMAP expression levels may serve as an indicator of vascular dysfunction and a potential novel therapeutic target for PPAR-γ ligands and other to-be-identified drugs. Further studies are required to determine if the putative link between SLMAP expression and endothelial dysfunction can be extended to the vasculature of other animal models of diabetes and humans with diabetes. Furthermore, the data here suggest that cellular mechanisms linking SLMAP proteins to endothelial function are worthy of further investigation in view of the critical role of the endothelium in vascular biology (13).

GRANTS

This study was supported by a Research Infrastructure grant from the Royal Melbourne Institute of Technology (RMIT) University (to H. Ding and C. R. Triggle), a Faculty of Life Sciences research grant from the RMIT University (to H. Ding), a Heart and Stroke Foundation of Ontario operating grant (to B. S. Tuana), a Canadian Institutes of Health Research grant (to D. L. Severson), a Canadian Diabetes Association operating grant (to T. J. Anderson.
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