Membrane Abnormalities of Vascular Smooth Muscle of Mesenteric Arteries of Spontaneous Diabetic BB Rats

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Abstract

Mesenteric arteries were isolated from the spontaneous diabetic BB rats, non-diabetic BB rats and regular Wistar control rats. Gross morphology indicated that the mesenteric vascular bed of the control Wistar rats had a normal development of mesenteric fat pad around the vessels, while that of the diabetic BB rats showed drastically reduced perivascular fat pad, suggesting greater mobilization of fat for energy consumption in the hyperglycemic state of diabetes mellitus. The perivascular mesenteric fat pad of the non-diabetic BB rats was intermediate between those of the Wistar control and diabetic BB rats. The wet weight of the mesenteric arteries following removal of fat, vein and connective tissues was significantly greater in diabetic BB rats than in the corresponding controls. Microsomal membranes isolated from the mesenteric arteries of diabetic BB rats showed increased alkaline phosphatase and 5'-nucleotidase activities compared to those isolated from the two groups of non-diabetic control rats. Acid phosphatase activities were higher in both BB rat groups compared to the Wistar group. The total Ca\(^{2+}\) uptake by the microsomes of mesenteric arteries in the presence of ATP was not different among three experimental groups, but the ATP-dependent active transport of Ca\(^{2+}\) was significantly increased and the passive Ca\(^{2+}\) binding was significantly reduced in diabetic group compared to the other two non-diabetic groups. Our results demonstrate that in the spontaneously diabetic BB rats, alterations in both structural and functional parameters may underline the vascular complications associated with type I diabetes mellitus in humans.

Key words: Mesenteric artery, microsomal membranes, calcium, diabetes mellitus, alkaline phosphatase, 5'-nucleotidase

Introduction

Cardiovascular complications account for a large proportion of all diabetic deaths. It has been long recognized that altered vascular reactivity, frequently found in sustained hypertension, is also present in diabetes mellitus (Sakai and Kwan, 1993). Some of the changes in
vascular reactivity in diabetes are endothelium-dependent (Meraji et al., 1987; Durante et al., 1988; Heygate et al., 1995; Pieper et al., 1997) and some are not (Sullivan and Sparks, 1979; While and Carrier, 1990; Inazu et al., 1991). Clinical findings indicated that elevation of arterial blood pressure was present in 40-80% of the diabetic patients (Yong et al., 1998; Pavlovic et al., 1999). In animal studies, there was a dispute about the development of hypertension following the induction of diabetes mellitus (Kawashima et al., 1978; Jackson and Carrier, 1981; Kusaka et al., 1987; Hicks et al., 1998). Similar structural changes of vascular tissues, such as vascular hypertrophy (Larson and Haudenschild, 1988; Bohlen and Larsh, 1991) and reduced elastin contents (Kwan et al., 1982) have been observed in hypertension and diabetes. Biochemical correlates of these structural changes, such as the increased activities of vascular muscle elastase, were similarly observed in the aortas of diabetic (Kwan et al., 1982) and hypertensive (Ito et al., 1987) rats.

Since Ca$^{2+}$ is a ubiquitous cellular messenger in various cell functions, alterations in Ca$^{2+}$ handling by membranes from different tissues in diabetic animals would be of special relevance to the etiology of diabetogenesis as well as associated complications. Diabetes mellitus in fact has been considered as a disease of abnormal cellular Ca$^{2+}$ metabolism (Levy et al., 1994). Abnormal Ca$^{2+}$ balance and deranged vitamin D metabolism are known complications of diabetes and may account for altered Ca$^{2+}$ transport in kidney, intestinal mucosa and bone (Nyomba et al., 1989). However, despite repeated findings of reduced Ca$^{2+}$ transport by vascular muscle membranes in rats with different models of hypertension (Kwan et al., 1980; Kwan, 1989), it is not clear whether similar changes in Ca$^{2+}$ handling by membranes from vascular smooth muscle are present in diabetes mellitus. Furthermore, much of the work on vascular changes in diabetes mellitus was performed using larger arteries, such as aorta (Sullivan and Sparks, 1979) and renal artery (Inazu et al., 1991). There have been no studies on the membrane biochemistry of the vascular smooth muscle of mesenteric arteries from the spontaneously diabetic rats. Therefore, the purpose of this communication is to report our findings on the physical characteristic of mesenteric arteries and some biochemical parameters of the isolated microsomal membranes of the vascular muscle isolated from spontaneous diabetic BB rats.

**Methods**

*Experimental animals*

Male Wistar rats (5-6 months old) were purchased from Charles River (New York). The spontaneous diabetic BB rats as well as the corresponding non-diabetic control rats (from the same colony of the BB rats but failed to elicit hyperglycemia) of the same age were supplied by Dr. P. Thibert, Animal Resources Division of Sir Frederick G. Banting Research Centre (Ottawa). These diabetic BB rats were diabetic upon arrival from Ottawa and were titrated daily with s.c. insulin injection according to standard instructions to maintain the blood glucose level in the range of 300-400 mg/dl. Rats were maintained in the animal facilities of McMaster University Health Sciences Centre under standard laboratory conditions. Non-diabetic BB rats with plasma glucose level $>200$ mg/ml at the time of experiment were not included.
Vascular changes in diabetic mellitus

Measurement of blood pressure and plasma glucose

Systolic blood pressure as well as the heart rate of all rats were measure using by tail-cuff method (Kwan et al., 1980) 1 or 2 days before the day of experiment. Blood glucose levels were measured in deproteinized plasma samples by the glucose-oxidase method (Kwan et al., 1982) regularly and 1 or 2 days before the day of the experiment.

Handling of tissues

On the day of experiment, all three groups of rats (the diabetic BB rats, the non-diabetic control rats and the normal Wistar control rats), 6-8 rats per group, were stunned by a blow on the head and immediately killed by cerebral dislocation. The heart and mesenteric vascular bed were removed from each individual rat, blotted dry on filter papers, weighed and dissected to separate the left ventricles and mesenteric arteries from the bulk vascular tissues (Kwan et al., 1979).

Preparation and characterization of microsomal membrane fractions

Tissues from 6-8 rats were pooled in each group and all three groups were simultaneously processed in the same experiment. The vascular tissues in each group were minced and homogenized in ice-cold imidazole (10 mM, pH 7.4)/sucrose (250 mM) solution. The homogenates were subject to a series of differential centrifugation to step-wise remove tissue debris and nuclei at 800×g for 10 min (to obtain the post-nuclear supernatant), mitochondrial fragments (10,000×g for 10 min) and soluble fractions (55,000×g for 30 min) to finally obtain the microsomal membrane fractions, which contain primarily membrane vesicles capable of active transport of Ca²⁺ (Kwan 1989). Methods for the determination of various membrane marker enzyme activities used to characterize the membrane composition have previously been reported in detail (Kwan et al., 1979; Kwan, 1989). Membrane protein was measured spectrophotically at 595 nm using the commercial kit (Bio-Rad, Hercules, CA).

Measurement of Ca²⁺ accumulation by microsomal membranes

Total accumulation of Ca²⁺ by microsomal membranes was performed by Millipore filtration technique (Kwan et al., 1970; 1980) in solutions containing 5 mM ATP and 17 μM free Ca²⁺ in 250 mM sucrose/10 mM imidazole (pH 7.2, 37°C) containing trace amount of ⁴⁰Ca²⁺ and 10 μM sodium azide (to inhibits Ca²⁺ accumulation by the contaminating mitochondrial fragments) over 10 min reaction time. Ca²⁺ binding was performed in a parallel fashion in the absence of ATP. The ATP-dependent Ca²⁺ accumulation, calculated as the difference between the total Ca²⁺ accumulation in the presence of ATP and the Ca²⁺ binding in the absence of ATP, has previously been shown to be inhibited by Ca²⁺ ionophore, A23187, indicative of an active Ca²⁺ transport (Kwan et al., 1979).

Statistical analysis

All data are expressed as means±SEM and were analyzed by two-tailed unpaired Students t-test for the parameters obtained from individual rats and by paired t-test when comparisons were made between parameters obtained with microsomal membranes, which were prepared
from pooled tissues in all three groups and performed in paralleled fashion.

Results and discussion

Physiological parameters of diabetic and control rats

The physical characteristics and relevant cardiovascular parameters for all three groups of rats used in four separate experiments in this study are shown in Table 1. The diabetic rats showed consistently lower body weight compared to the rats in the other two non-diabetic groups. Although the blood glucose of diabetic rats was always highly elevated and needs to be controlled to the level of 300-400 mg/dl for survival with daily insulin treatment, the systolic blood pressure remained normal showing slightly higher but not statistically different from those of the other two non-diabetic groups. There have been a number of disputes on the hypothesis that rats with experimentally induced diabetes mellitus are associated with elevation of blood pressure (Kawashima et al., 1978; Jackson and Carrier, 1981; Kusaka et al., 1987; Hicks et al., 1998). However, our results show that the systolic blood pressure, like the heart rate, of the diabetic BB rats was slightly but not significantly higher than that of the control rats. This finding is in accord with an earlier observation in spontaneously diabetic rats (Krizsan-Agbass and Bunag, 1971), in which however an enhanced reflex tachycardia was

Table 1. Physiological parameters of the rats used in this study.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Number of rats</th>
<th>Age (days)</th>
<th>Body weight (grams)</th>
<th>Plasma glucose (mg/dl)</th>
<th>Systolic blood pressure (mmHg)</th>
<th>Heart rate (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Wistar rats</td>
<td>30</td>
<td>158±16</td>
<td>472±45</td>
<td>125±20</td>
<td>121±10</td>
<td>374±34</td>
</tr>
<tr>
<td>Non-diabetic BB rats</td>
<td>28</td>
<td>163±15</td>
<td>452±53</td>
<td>120±23</td>
<td>126±12</td>
<td>375±39</td>
</tr>
<tr>
<td>Diabetic BB rats</td>
<td>26</td>
<td>156±21</td>
<td>363±38*</td>
<td>333±60*</td>
<td>131±18</td>
<td>375±49</td>
</tr>
</tbody>
</table>

Note. Data are expressed as means±SEM from the number of rats indicated in each group. *p<0.05 vs control Wistar rats (*) and non-diabetic BB rats (*).
Changes of mesenteric vascular bed and tissue wet weight in diabetes

A lower body weight has repeatedly been observed in experimental diabetes mellitus (Sullivan and Sparks, 1979; White and Carrier, 1990; Inazu et al., 1991) and is also characteristic of spontaneous diabetes mellitus in rats (Kwan et al., 1982), as also confirmed in this work. This is presumably due to the metabolic disturbance as a result of persistent hyperglycemia, in which the plasma glucose was not effectively utilized as the energy source resulting in the breakdown of fat and protein as alternative source of energy. The substantial loss of the mesenteric vascular fat pad as shown in Fig. 1 provided further support for the accelerated breakdown of fat. Indeed, epididymal fat pad was also found to be reduced in the diabetic group compared to the control groups (not shown). Table 2 shows the summary of the data on the wet weight of mesenteric tissues and ventricles. Despite the reduced wet weight of the whole mesenteric vascular bed of the diabetic group, the wet weight of the trimmed mesenteric arteries was significantly more in the diabetic group compared to the non-diabetic Wistar control rats. It is interesting to note that, in both Fig. 1 and Table 2, the corresponding parameters for the non-diabetic BB rats were intermediate between the diabetic group and the Wistar control group despite the fact that we have excluded the rats which developed intermediate level of plasma glucose concentration (>200 mg/dl but <300 mg/dl) at the time of experiment. Since the non-diabetic BB rats were from the same original colony for selective breeding of the diabetic BB rats, these diabetes-prone BB rats may have the genetic predisposition for the metabolic disturbance even if their blood glucose levels remain normal at the time of experiment. The heart weight and left ventricle weight was slightly lower in diabetic BB rats compared to the non-diabetic control rats, but were not significantly different among these three groups when the cardiac tissue wet weight was normalization by the body weight or when the ventricular weight was normalized by the heart weight. This observation suggests a lack of ventricular hypertrophy frequently found to be associated with hypertension, and is consistent with the finding of unaltered systolic blood pressure and heart rate.

Membrane associated enzymatic activities

The NADPH-cytochrome c reductase activities, which reflect the amount of the sarcoplasmic

<table>
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<th>Table 2. Tissue wet weight of mesenteric vascular bed and heart.</th>
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<tr>
<td>Experimental groups</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Control Wistar rats</td>
</tr>
<tr>
<td>Non-diabetic BB rats</td>
</tr>
<tr>
<td>Diabetic BB rats</td>
</tr>
</tbody>
</table>

Note. Data are expressed as means±SEM from 7-8 rats in each group. *p<0.05 vs Wistar control rats and bp<0.05 vs non-diabetic BB rats by unpaired t-test.
mic reticulum vesicles in the microsomal fraction and the cytochrome c oxidase activities, which reflect the amount of contaminating mitochondrial fragments (Kwan et al., 1979; Kwan, 1989), were not statistically different among these three groups. The Mg\(^{2+}\)-ATPase activities and phosphodiesterase I activities, the plasma membrane enzyme activities (Kwan, 1982; Kwan, 1989), both of which were similarly enriched 5 fold in the microsomal fraction over the post-nuclear supernatant, and also not different amount these groups. These results suggest that the microsomal fractions from these three groups were enriched in the plasma membranes to a similar extent without differential contamination by the mitochondria or sarcoplasmic reticulum. However, another set of plasma membrane associated enzymes, alkaline phosphatase, and 5′-nucleotidase, both of which were similarly enriched by about 3 fold in microsomal fractions over the post-nuclear supernatant, showed enhanced activities in diabetic BB rats compared to non-diabetic BB rats and the Wistar control rats. We speculate that these plasmalemmal enzymes may be present in a micro-domain of the plasma membranes, such as caveolae, that contain many glycosylphosphatidylinositol–anchored proteins, including 5′-nucleotidase and alkaline phosphatase (Anderson, 1998). The role of these enzymes in the vascular muscle is not clear, but their possible role in protein synthesis and tissue regeneration has long been suggested (Zemplenyi, 1968). Indeed, elevated activities of alkaline phosphatase and 5′-nucleotidase activities of rat mesenteric artery have been found to be associated with hypertrophied vascular tissues in hypertension (Kwan et al., 1980; Kwan, 1989). Although the rats used in this study were not hypertensive, the present results show strong indication that the mesenteric arteries were hypertrophied in diabetic BB rats compared to both non-diabetic control rats. It is interesting to note that, following the onset of diabetes, diabetic BB rats showed a striking elevation of the serum alkaline phosphatase activities, which were positively correlated with blood glucose level (Scott et al., 1984).

Table 3. Enzymatic activities of microsomal membranes isolated from mesenteric arterial smooth muscle

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Control Wistar rats</th>
<th>Non-diabetic BB rats</th>
<th>Diabetic BB rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH cytochrome c reductase</td>
<td>0.80±0.22</td>
<td>0.72±0.10</td>
<td>0.99±0.45</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>3.57±2.09</td>
<td>2.61±1.33</td>
<td>2.72±1.38</td>
</tr>
<tr>
<td>Mg(^{2+})-ATPase</td>
<td>243±86</td>
<td>235±71</td>
<td>249±85</td>
</tr>
<tr>
<td>Phosphodiesterase I</td>
<td>12.4±5.1</td>
<td>14.9±7.5</td>
<td>14.8±4.5</td>
</tr>
<tr>
<td>5′-Nucleotidase</td>
<td>13.0±3.6</td>
<td>12.1±2.4</td>
<td>20.0±2.1(^{ab})</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>159±48</td>
<td>193±48</td>
<td>318±53(^{ab})</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>69±14</td>
<td>92±11(^a)</td>
<td>97±7(^a)</td>
</tr>
</tbody>
</table>

Note. The data are expressed as means±SEM from 4 separate paired experiments using pooled tissues from 6-8 animals each. \(^a\)p<0.05 vs control Wistar rats and \(^b\)p<0.05 vs non-diabetic BB rats by paired t-test.

NADPH cytochrome c reductase and cytochrome c oxidase activities were expressed as changes of absorption of cytochrome c measured at 550 nm per min per mg of protein. Mg\(^{2+}\)-ATPase and 5′-nucleotidase activities were expressed as formation of inorganic phosphate per hour per mg protein. Alkaline and acid phosphatase activities were expressed as changes of optical density at 400 nm per min per mg protein using p-nitrophenyl phosphate as the substrate. \(^a\)Significantly different from the Wistar control rats. \(^ab\)Significantly different from the non-diabetic BB rats.
The acid phosphatase activity, which was, like alkaline phosphatase activity, also similarly enriched 3 fold in microsomal fractions of all three groups, was elevated in both groups of diabetic and non-diabetic BB rats compared to the Wistar control rats. Acid phosphatase has conventionally been regarded as a lysosomal marker enzyme, but it may also be present in the plasma membranes of vascular smooth muscle (Kwan and Ito, 1987). Unlike alkaline phosphatase activity, which was elevated only in the diabetic group, elevated acid phosphatase activity was found in both diabetic and non-diabetic groups of BB rats. An earlier study using rabbit aorta in experimental diabetes showed a reduced level of acid phosphatase activity (Wolinski et al., 1978), but a more recent study using rat aorta showed an elevated acid phosphatase activity in experimental diabetes (Kobayashi et al., 1998). It is not known whether or not the different findings are due to species difference (Kwan and Ito, 1987). The elevated acid phosphatase activity in the smooth muscle membranes of mesenteric arteries of the spontaneously diabetic rats may also, at least in part, be indicative of the underlying genetic characteristics in diabetes-prone BB rats not necessarily associated with the complications of hyperglycemia.

Changes of $\text{Ca}^{2+}$ handling by vascular smooth muscle microsomal membranes in diabetes

We have also observed abnormalities in the $\text{Ca}^{2+}$ handling by microsomal fractions of mesenteric arteries isolated from diabetic BB rats. Table 4 shows that the total $\text{Ca}^{2+}$ uptake in the presence of ATP remained practically, unaltered in diabetes, but the binding of $\text{Ca}^{2+}$ to the microsomal membranes in the absence of ATP was significantly higher in diabetes. This is also paralleled by a significantly decreased ATP-dependent $\text{Ca}^{2+}$ transport across the microsomal membrane in the diabetic group compared to either of the non-diabetic groups. The reduced ATP-dependent $\text{Ca}^{2+}$ transport activity is unlikely to be due to increased breakdown of ATP by membranes from diabetic rats, since the $\text{Mg}^{2+}$-ATPase activities was the same in the diabetic and control groups. We have previously reported (see review Kwan, 1989) that ATP-dependent $\text{Ca}^{2+}$ transport by microsomal membrane vesicles of mesenteric arteries of rats with spontaneous or experimental hypertension was diminished compared to that of normotensive control rats. However, in hypertension, the $\text{Ca}^{2+}$ binding to the arterial smooth muscle membranes was unaltered. It is possible that the reduced ATP-dependent $\text{Ca}^{2+}$ transport in spontaneous diabetes mellitus may represent an increased membrane permeability to $\text{Ca}^{2+}$ due to injuries to membranes and an increased $\text{Ca}^{2+}$ binding may represent an adaptive

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Total $\text{Ca}^{2+}$ accumulation</th>
<th>$\text{Ca}^{2+}$ binding</th>
<th>ATP-dependent $\text{Ca}^{2+}$ uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Wistar rats</td>
<td>8.3±0.8</td>
<td>1.7±0.3</td>
<td>6.5±0.3</td>
</tr>
<tr>
<td>Non-diabetic BB rats</td>
<td>7.4±1.1</td>
<td>1.9±0.4</td>
<td>5.6±1.1$^a$</td>
</tr>
<tr>
<td>Diabetic BB rats</td>
<td>8.2±0.6</td>
<td>3.3±0.8$^{ab}$</td>
<td>5.1±1.0$^{ab}$</td>
</tr>
</tbody>
</table>

Note. Data are expressed as means±SEM of 4 separate paired experiments. $^a$p<0.05 vs control Wistar rats. $^b$p<0.05 vs nondiabetic rats. ATP-dependent $\text{Ca}^{2+}$ uptake represents the difference between the total $\text{Ca}^{2+}$ accumulation and $\text{Ca}^{2+}$ binding, all expressed as μmoles of $^{45}\text{Ca}^{2+}$ accumulated per g protein in 10 min.
change to stabilize the membranes. This could be mediated via altered lipid metabolism in diabetes mellitus through the modification of the phospholipids of the membranes leading to enhanced binding of Ca\(^{2+}\) to the phospholipids. Alternatively, the reduced ATP-dependent Ca\(^{2+}\) transport may also be the result of reduced activity or number of Ca\(^{2+}\)-ATPase pump sites. Differentiation of these alternative explanations will require more detailed future investigations.

**General conclusions**

We have, for the first time, characterized the physical and biochemical properties of the mesenteric arteries isolated from rats with spontaneous diabetes mellitus. The mesenteric vascular bed from diabetic rats was characterized by a prominent reduction of perivascular fat pad, indicative of fat being mobilized as the major source for the cellular energy, and increased arterial muscle mass, indicative of tissue proliferation. Elevated alkaline phosphatase and 5'-nucleotidase activities are consistent with the regenerative/proliferative process of the vascular tissue in response to tissue injury inflicted by the disease. This is also supported by the finding of elevated acid phosphatase activity, which in part suggests cellular degenerative process involving lysosomes. Increased Ca\(^{2+}\) binding may also promote calcification of vascular tissues leading to atherosclerosis, whereas reduced ATP-dependent Ca\(^{2+}\) transport may be associated with compromised contractile function of the vascular smooth muscle frequently found to be associated with diabetes mellitus unrelated to hypertension. The hypertrophied vascular tissue and elevated alkaline phosphatase and 5'-nucleotidase activities, although closely associated with hypertensive diseases, are apparently not sufficient to cause hypertension in diabetic mellitus. These results are useful in providing the fundamental basis for future investigation of the molecular mechanisms leading to the structural and functional changes in vascular tissues associated with diabetes mellitus.

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**References**


Vascular changes in diabetic mellitus


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