Minalrestat, an Aldose Reductase Inhibitor, Corrects the Impaired Microvascular Reactivity in Diabetes

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ABSTRACT

We demonstrated that aldose reductase inhibition corrects the impaired microvascular responses to inflammatory mediators in diabetic rats. To study the mechanism involved in the restoring effect of aldose reductase inhibition, we examined the effects of minalrestat, another aldose reductase inhibitor, on the responses of mesenteric microvessels studied in vivo to permeability-increasing agents in diabetic and galactosemic rats. The diabetic group was treated from 3 days after the alloxan injection with minalrestat (10 mg/kg/day) for 30 days and the minalrestat treatment (10 mg/kg/day/7 days) of galactosemic rats started concomitantly with the induction of galactosemia. The mesenteric microvessel reactivity was studied using intravital microscopy and changes in vessel diameters were estimated after the topical application of vasoactive agents. The impaired responses to bradykinin, histamine, and platelet-activating factor of arterioles and venules observed in diabetic and galactosemic rats were completely prevented by minalrestat. Neither diabetes nor galactosemia affected responses to acetylcholine and sodium nitroprusside. Responses to these agents were not modified by aldose reductase inhibition. The restoring effect of minalrestat was reversed by inhibition of nitric oxide (NO) synthesis with Nω-nitro-L-arginine methyl ester, by blocking K+ channel with tetraethylammonium but not by cyclooxygenase inhibition with diclofenac. Therefore, we concluded that NO, membrane hyperpolarization, but not cyclooxygenase products are involved in the beneficial effect of minalrestat on the microvascular reactivity in diabetes. Together, these findings led us to suggest that aldose reductase inhibition might ameliorate diabetic complications through the correction of the altered microvascular reactivity by a mechanism that involves NO and membrane hyperpolarization.

Aldose reductase is the first and rate-limiting enzyme in the polyol pathway and reduces the aldehyde form of glucose to sorbitol. Several experimental and clinical studies have suggested a link between the increased polyol pathway activity and the occurrence of chronic diabetic complications. Strict glycemic control, such as with intensive insulin therapy, was the only method believed to delay the progression of chronic diabetic complications (American Diabetes Association, 1993; Santiago, 1993). The aldose reductase inhibitors (ARIs) are a new class of drugs aimed at the control of the consequences of hyperglycemia rather than at the control of hyperglycemia per se. Several studies demonstrated that nerve function (Kamijo et al., 1993; Cameron et al., 1996), aspects of nephropathy (Chang et al., 1991), retinopathy (Kinoshita et al., 1984; Robison, 1988), as well as defective leukocyte-endothelial interaction (Cruz et al., 2000) and vascular dysfunction (Cameron and Cotter, 1992; Tesfamariam et al., 1993; Otter and Chess-Williams, 1994; Fortes et al., 1996) are ameliorated or prevented by treatment of diabetics with aldose reductase inhibitor.

Functional changes in the behavior of microvessels are observed in diabetes mellitus (Garcia-Leme, 1981, 1989). Decreased responses of mesenteric microvessels to histamine, bradykinin and platelet-activating factor (PAF), vasodilators with permeability-increasing properties, are observed in alloxan-diabetic rats (Fortes et al., 1983a,b, 1984, 1989) without any alteration to acetylcholine and sodium nitroprusside. The fact that aldose reductase is involved in such alteration is demonstrated by the observation that similar diabetic-like vascular dysfunction occurs in the galactosemic state and is restored by tolrestat, an aldose reductase inhibitor (Fortes et al., 1996).

In several clinical studies the effects of the aldose reductase inhibitors, most notably tolrestat, sorbinil, ponalrestat, epalrestat, and imirestat, on chronic symptomatic diabetic
neuropathy (Pitts et al., 1986; Boulton et al., 1990; Kirchain and Rendell, 1990; Stribling, 1990; Brazzell et al., 1991; Florkowski et al., 1991) were demonstrated; however, these inhibitors were withdrawn from clinical trials due to toxicity or lack of efficacy (Malamas et al., 1994).

Orally active aldose reductase inhibitors are limited for the most part to two classes, the carboxylic acids (tolrestat) and the cyclic imides (minalrestat). We demonstrated that aldose reductase inhibition with tolrestat corrects the impaired responses to inflammatory mediators in diabetic rats (Fortes et al., 1996). However, the mechanism involved in the restoring effect of aldose reductase inhibition remained to be studied. Therefore, in the present study we investigated the effect of minalrestat on the responses to vasoactive agents of mesenteric microvessels studied in vivo in situ in alloxan-diabetic and galactosemic rats and the mechanism(s) involved in it.

**Materials and Methods**

The experimental protocols were approved and performed in accordance with the guidelines of the Institute of Biomedical Sciences Committee.

Animals. Male Wistar rats (weighing 170 to 190 g at the beginning of the experiments) were obtained from our breeding colony at the Institute and were randomized into six groups that were aged- and weight-matched. All animals were housed under the same conditions and had food and water ad libitum. The groups consisted of the following: 1) saline-treated diabetic rats, 2) saline-treated nondiabetic control rats, 3) diabetic rats treated with minalrestat for 30 days; 4) nondiabetic control rats treated with minalrestat for 30 days; 5) saline-treated galactosemic rats; 6) saline-treated nongalactosemic control rats; 7) galactosemic rats treated with minalrestat for 7 days; and 7) nongalactosemic control rats treated with minalrestat for 7 days. Rats of the saline-treated groups received the same volume of saline by the same route and for the same period as the galactosemic rats and the mechanism(s) involved in it.

In all of the treated groups, minalrestat was suspended in saline with 2% Tween 80 and was administered at a dose of 10 mg/kg daily by gavage. The effectiveness of this treatment was previously demonstrated in our laboratory by a high level of polyol pathway blockade in acetic nerve of diabetic rats (J. W. C. M. Cruz, M. W. Soto-Suazo, T. C. Hohman, E. H. Akamine, T. T. Zorn, and Z. B. Fortes, unpublished data).

**Induction of Diabetes and Galactosemia.** Diabetes mellitus was induced with an injection of alloxan (40 mg/kg i.v.) dissolved in physiological saline. Control rats were injected with physiological saline alone. On day 3, a tail vein blood sample was removed for blood glucose concentrations above 11.0 mM were determined with a blood glucose monitor. Minalrestat treatment started 3 days after alloxan or saline injection.

Galactosemia was induced by feeding animals with a diet containing 50% galactose for 7 days. Minalrestat treatment of galactosemic rats started concomitantly with the induction of galactosemia. Control rats received ground chow and minalrestat treatment in the same period as the galactosemic rats.

**Characterization of the Diabetic and Galactosemic Rats.** Control, diabetic, and aldose reductase inhibitor-treated diabetic rats were placed in a metabolic cage during 24 h to evaluate the food and water consumption and the urine volume. Glycosuria was qualitatively assessed in urine with the aid of reagent strips.

**Procedures with Mesenteric Microvessels in Situ.** The animals were anesthetized with a subcutaneous injection of chloral hydrate (450–500 mg/kg). The mesentery was exteriorized and arranged for microscopic observation according to Zweifach (1948) with slight modifications (Fortes et al., 1984). The animals were kept on a special board heated at 37°C, which included a transparent plate on which the tissue to be transilluminated was placed. The mesenteric preparations were maintained moist and warmed throughout the experiment by bathing the tissue with warmed Ringer-Locke’s solution (pH 7.2–7.4) containing 1% gelatin. The composition of the solution was 154.0 mmol/l NaCl, 5.6 mmol/l KCl, 2.0 mmol/l CaCl2·2H2O, 6.0 mmol/l NaHCO3, and 5.5 mmol/l glucose. A 500-line television camera (JVC, Tokyo, Japan) was combined with a trinocular microscope to facilitate observation of the enlarged image.

**TABLE 1**

Characteristics of the diabetic rats treated with saline or minalrestat 10 mg/kg/day for 30 days and their respective controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Glycosuria</th>
<th>Urine Volume</th>
<th>Food Intake</th>
<th>Water Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/dl</td>
<td>ml/24 h</td>
<td>g/24 h</td>
<td>ml/24 h</td>
</tr>
<tr>
<td>Saline-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Undetectable</td>
<td>4.25 ± 1.1</td>
<td>19.7 ± 5.8</td>
<td>42.5 ± 7.8</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>≥1000***</td>
<td>57.6 ± 4.5***</td>
<td>46.0 ± 4.8*</td>
<td>137.5 ± 4.0***</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>Minalrestat-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Undetectable</td>
<td>4.9 ± 1.4</td>
<td>16.0 ± 6.4</td>
<td>33.6 ± 9.5</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>≥1000***</td>
<td>62.8 ± 7.4***</td>
<td>38.0 ± 4.5*</td>
<td>123.8 ± 12.1***</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05 and ***P < 0.001 compared with saline- and minalrestat-treated controls.

**TABLE 2**

Characteristics of the galactosemic rats treated with saline or minalrestat 10 mg/kg/day for 7 days and their respective controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Glycosuria</th>
<th>Urine Volume</th>
<th>Food Intake</th>
<th>Water Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/dl</td>
<td>ml/24 h</td>
<td>g/24 h</td>
<td>ml/24 h</td>
</tr>
<tr>
<td>Saline-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Negative</td>
<td>3.4 ± 0.8</td>
<td>17.6 ± 5.3</td>
<td>36.0 ± 9.8</td>
</tr>
<tr>
<td>Galactosemic</td>
<td>100.0***</td>
<td>81.2 ± 17.8**</td>
<td>37.6 ± 4.1*</td>
<td>125.6 ± 21.4***</td>
</tr>
<tr>
<td>Minalrestat-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Negative</td>
<td>3.0 ± 0.8</td>
<td>15.4 ± 5.2</td>
<td>28.4 ± 8.6</td>
</tr>
<tr>
<td>Galactosemic</td>
<td>250.0***</td>
<td>87.6 ± 21.5**</td>
<td>37.8 ± 4.5*</td>
<td>128.4 ± 22.4**</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, and ***P < 0.001 compared with saline- and minalrestat-treated controls.
Body weight gain in alloxan-diabetic (33.6 ± 7.1 g; n = 15) and minalrestat-treated diabetic (40.7 ± 7.8 g, n = 19) rats were significantly less (P < 0.05) compared with age-matched control animals (136.8 ± 5.1 g, n = 18). Blood glucose concentrations were found to be similarly elevated in saline-treated diabetic (19.7 ± 0.6 mM; n = 22) and minalrestat-treated diabetic groups (20.1 ± 0.8 mM, n = 24) (P < 0.001) compared with age-matched control animals (4.9 ± 0.4 mM, n = 20). Rats fed a 50% galactose diet for 7 days lost weight (−10.4 ± 2.8 g, n = 7) (P < 0.001) compared with rats who were fed with the regular diet and gained weight (+33.5 ± 3.8 g, n = 6). Minalrestat treatment did not affect body weight or glucose levels in diabetic rats.

Results

General Characteristics. Body weight gain in alloxan-diabetic (33.6 ± 7.1 g; n = 15) and minalrestat-treated diabetic (40.7 ± 7.8 g, n = 19) rats were significantly less (P < 0.05) compared with age-matched control animals (136.8 ± 5.1 g, n = 18). Blood glucose concentrations were found to be similarly elevated in saline-treated diabetic (19.7 ± 0.6 mM; n = 22) and minalrestat-treated diabetic groups (20.1 ± 0.8 mM, n = 24) (P < 0.001) compared with age-matched control animals (4.9 ± 0.4 mM, n = 20). Rats fed a 50% galactose diet for 7 days lost weight (−10.4 ± 2.8 g, n = 7) (P < 0.001) compared with rats who were fed with the regular diet and gained weight (+33.5 ± 3.8 g, n = 6). Minalrestat treatment did not

TABLE 3
Increase (%) in microvessel diameter induced by acetylcholine and sodium nitroprusside in diabetic rats treated with saline or minalrestat 10 mg/kg/day for 30 days and their respective controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Arterioles</th>
<th>Venules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetylcholine</td>
<td>Sodium Nitroprusside</td>
</tr>
<tr>
<td>Saline-treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.2 ± 2.4</td>
<td>11.9 ± 0.8</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 8)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>12.5 ± 1.0</td>
<td>9.6 ± 0.9</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 9)</td>
<td></td>
</tr>
<tr>
<td>Minalrestat-treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.6 ± 1.2</td>
<td>11.4 ± 1.9</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 9)</td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>12.5 ± 1.5</td>
<td>9.8 ± 0.6</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 13)</td>
<td></td>
</tr>
</tbody>
</table>
correct the body weight loss of galactosemic rats ($-11.1 \pm 3.0$ g, $n = 7$). Food and water intake, urine volume, and glycosuria were increased in diabetic (Table 1) and galactosemic (Table 2) rats compared with respective control rats. Minalrestat treatment did not correct these metabolic alterations.

**Response of Mesenteric Microvessels in Situ.** At resting conditions, there were no differences in the diameter (micrometers) of comparable types of vessels, either in saline-treated diabetic (23.6 $\pm$ 1.3, $n = 20$ and 30.5 $\pm$ 1.5, $n = 15$), arterioles and venules, respectively) and their respective controls (21.3 $\pm$ 0.8, $n = 16$ and 29.7 $\pm$ 1.8, $n = 12$) or in minalrestat-treated diabetic (21.6 $\pm$ 1.1, $n = 21$ and 28.1 $\pm$ 0.9, $n = 18$) rats. Values of diameter of arterioles and venules in galactosemic (21.2 $\pm$ 0.5, $n = 5$ and 36.5 $\pm$ 1.1, $n = 5$), minalrestat-treated galactosemic (21.2 $\pm$ 0.7, $n = 8$ and 38.8 $\pm$ 1.4, $n = 8$) and respective controls (20.9 $\pm$ 1.3, $n = 6$ and 32.9 $\pm$ 2.2, $n = 5$) rats were not significantly different.

Impaired responses of arterioles and venules to bradykinin, histamine, and PAF were observed in diabetic (33 days) rats. Minalrestat treatment (10 mg/kg/day) restored the decreased responses to these agents (Fig. 1).

Acetylcholine and sodium nitroprusside (Table 3) responses were not altered in diabetic rats. Minalrestat treatment did not interfere with the response to these agents either in control or diabetic animals.

Similarly to that found in diabetic rats, galactosemic rats exhibited reduced arteriolar and venular responses to histamine, bradykinin, and PAF. Minalrestat treatment corrected the impaired responses to these agents (Fig. 2).

There were no differences in the responses to acetylcholine and sodium nitroprusside (Table 4) between galactosemic and control rats. Minalrestat treatment did not alter these responses.

The restored responses to bradykinin (Fig. 3), histamine (Fig. 4), and PAF (Fig. 5) by minalrestat were inhibited in arterioles and venules of diabetic rats by L-NAME and TEA, but not by diclofenac.

**Discussion**

The results of this study are in agreement with our previous observations in which we demonstrated correction of the impaired response to inflammatory mediators by tolrestat, an ARI of carboxylic acid class, in diabetic rats (Fortes et al., 1996). In addition, we also demonstrated that the new ARI of
the cyclic imide class, minalrestat, corrected the decreased response to inflammatory mediators of mesenteric microvessels in diabetic rats. The responses of microvessels to acetylcholine and sodium nitroprusside (vasodilators not involved with the inflammatory response) were not altered by diabetes, galactosemia, or minalrestat treatment. The present findings, together with our previous work with tolrestat, allow us to suggest that the polyol pathway is involved in the impaired response to bradykinin, histamine, and PAF. The restoring effect of minalrestat was reversed by NO synthesis inhibition as well as by K⁺ channel blocking but not by cyclooxygenase inhibition.

There are previous studies on the beneficial effects of aldose reductase inhibitor treatment on the diabetic complications. Vascular dysfunction was corrected or prevented by treatment with different aldose reductase inhibitors in aorta (Tesfamariam et al., 1993; Otter and Chess-Williams, 1994) and mesenteric vascular bed (Keegan et al., 2000) of diabetic animals. Impaired relaxation to acetylcholine and calcium ionophore of aorta was corrected by ponalrestat in galactosemic rats (Cameron and Cotter, 1993). Inhibition of aldose reductase in vitro also corrected the decreased acetylcholine-induced relaxation of aorta incubated in elevated concentrations of glucose (Tesfamariam et al., 1993). However, most of these studies have been carried out in conduit arteries or microcirculation in vitro. In our study, we demonstrated no difference in the response to acetylcholine in diabetic and galactosemic rats studied in vivo. On the other hand, responses to inflammatory mediators were impaired in these animals and minalrestat corrected this alteration.
Minalrestat and Impaired Microvascular Reactivity

Aldose reductase inhibitor might correct the decreased response to inflammatory mediators in diabetic rats.

To understand the restoring mechanism of minalrestat, L-NAME, TEA, and diclofenac were used to investigate the possible participation of prostanoids, NO, and membrane hyperpolarization in this effect, respectively. Our data led us to suggest that NO and membrane hyperpolarization, but not prostanoids are involved in the restoration of impaired response to inflammatory mediators in diabetes by ARI, because diclofenac did not interfere with the effect of minalrestat, whereas L-NAME and TEA inhibited it. Therefore, although in diabetes the capacity of blood vessels to generate vasodilator prostaglandins, such as prostacyclin (Peredo et al., 1999), is decreased, the restoring effect of minalrestat seems to not involve an increase in products of cyclooxygenase activation.

Minalrestat might be interfering with the levels of NO. NADPH is an important cofactor for enzymes such as aldose reductase, NO synthase, cytochrome P450, and glutathione reductase (Dvornik, 1987; Ignarro, 1990; Quille et al., 1997). The activity of NADPH-requiring enzymes could be affected by depletion of NADPH due to the increased flux of glucose through the polyol pathway. Minalrestat could be restoring NADPH levels and as a consequence improve the NO generation, correcting the decreased response to inflammatory mediators. This hypothesis assumes that the depletion of NADPH in the aldose reductase reaction is functionally and spatially coupled to NADPH levels associated with NO synthase to be of biological relevance. Cytochrome P450 metabolites, cannabinoid-like substances, and K⁺, as well as NO are suggested to be the endothelium-derived hyperpolarizing factor (Félotou and Vanhoucke, 1999). The restoring effect of minalrestat could be related to Ca²⁺-activated K⁺ channels, because TEA, a blocker of this K⁺ channel, inhibited it. Impaired hyperpolarization-mediated responses had been observed in isolated mesenterics arteries (Fukao et al., 1997), renal microcirculation in vivo (De Vriese et al., 2000), and isolated perfused kidney (Fulton et al., 1996) in diabetes. Aldose reductase inhibitor treatment prevented partially the decreased endothelium-derived hyperpolarizing factor-mediated response of mesenteric vascular bed of diabetic rats (Keegan et al., 2000). Therefore, aldose reductase inhibitor could be improving the membrane hyperpolarization, facilitating the opening of K⁺ channels in diabetic rats. Although the vasodilator response to acetylcholine is also dependent on NO and membrane hyperpolarization, minalrestat might ameliorate the responses to mediators that have their responses impaired in diabetes.

In conclusion, we demonstrated that the mechanism by which minalrestat corrects the impaired responses to inflammatory mediators might involve membrane hyperpolarization and NO but not prostanoids.

Acknowledgments

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Fig. 5. Bar graphs showing percentage of increase in arteriole (A) and venule (B) diameter induced by platelet activating factor in saline-treated controls ( ), saline-treated diabetic ( ), and minalrestat-treated diabetic ( ); and the effects of treatment with diclofenac ( ), topical application of L-NAME ( ), and TEA ( ) on the response to bradykinin in minalrestat-treated diabetic. Data expressed mean ± S.E.M. * P < 0.05 compared with saline-treated control, minalrestat-treated diabetic, and minalrestat-treated diabetic with diclofenac. The number of rats per group is shown at the base of the columns.


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