Effects of seven days of galactose feeding and aldose reductase inhibition on mast cells and vessel morphometry in rat sciatic nerve

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Abstract

The association between mast cells and vessel morphometry in sciatic nerve was examined after seven days in animals fed a diet of 40% d-galactose and compared to control rats and to galactose-fed animals treated with the aldose reductase inhibitor, Tolrestat. Electron microscopy revealed an increase in the total number of mast cells and the number of degranulated mast cells in galactose-fed animals (7.8 ± 2.9; 2.6 ± 2.9; mean ± SD) compared to controls (4.6 ± 2.1; degranulated mast cells were not seen in any control nerves) and Tolrestat-treated, galactose-fed animals (4.4 ± 2.5; 0.1 ± 0.4). Although no significant differences were noted in the numbers of vessels between the three groups, an index of vasoconstriction was significantly increased in the galactose-fed animals (0.115 ± 0.048; mean ± SD) compared to controls (0.068 ± 0.011) and Tolrestat-treated, galactose-fed animals (0.075 ± 0.020). These data suggest that mast cell degranulation is associated with the vascular constriction induced by seven days of galactose intoxication and that both may be prevented by inhibiting aldose reductase.

Keywords: Aldose reductase; Galactose intoxication; Mast cells; Peripheral nerve; Vascular permeability; Vasoconstriction

1. Introduction

Mast cells are normal constituents of the peripheral nerve microenvironment (Olsson, 1968) and contain bioactive amines capable of increasing vascular permeability (Olsson, 1967; Powell et al., 1980; Gulati et al., 1985; Gulati et al., 1985; Carroll et al., 1988; Harvey et al., 1994). By light microscopy, mast cell number is increased following nerve trauma (Enerback et al., 1965) and in chronic experimental galactose neuropathy (Malmgren et al., 1979; Powell et al., 1981; Forcier et al., 1991). The galactose-induced increase in mast cell number is accompanied by edema and altered vascular permeability along with other aspects of nerve dysfunction including accumulation of polyol pathway products, myo-inositol depletion and nerve conduction velocity deficits (Sharma et al., 1976; Myers and Powell, 1984; Mizisin et al., 1986; Mizisin et al., 1990; Mizisin and Kalichman, 1993). All of these disorders are prevented by treatment with aldose reductase inhibitors (ARIs) that block flux through the first part of the polyol pathway (Robison, 1984; Wadhwani et al., 1989; Mizisin and Powell, 1993) and are currently in clinical trial for treatment of diabetic neuropathy.

Recently, an increase in degranulated mast cells after seven days of galactose feeding was observed by electron but not light microscopy (Kalichman et al., 1995). This increase in degranulated mast cells was accompanied by constriction of endoneurial vessels, increased water content, and increased vascular permeability, suggesting that mast cells may have a key role in the disturbance of the nerve microenvironment induced by galactose intoxication. Although ARIs can prevent the edema, increased mast cell number, and vascular permeability defects in nerve from galactose-fed rats (Wadhwani et al., 1989; Mizisin and Powell, 1993), it is not clear how this occurs and neither the effect of ARIs on degranulation of mast cells nor vessel constriction has been studied by quantitative electron microscopy. Therefore, the purpose of the present study was to examine the association between mast cells and endoneurial vessels by quantifying mast cell and ves-
2. Materials and methods

With approval of the San Diego Veterans Administration Animal Studies Subcommittee, thirty female Sprague-Dawley rats (250 g; Charles River, San Diego, CA) were randomly assigned to one of three groups. Insulin-independent hyperglycemia was induced in one group by feeding an ad lib diet containing 40% d-galactose by weight (Purina, Richmond, IN). The second group was fed a similar diet and treated with the ARI, Tolrestat (Wyeth-Ayerst, Philadelphia, PA; 50 mg/kg orally by gavage daily). Age-matched normal animals served as controls. All animals received diets containing 100% of required micronutrients and were housed with wire cage bottoms to decrease contact with urine and feces.

After seven days of hyperglycemia, rats were anesthetized by intraperitoneal injection (2 ml/kg) of pentobarbital (12.5 mg/ml) and diazepam (1.25 mg/ml) in bacteriostatic saline. Under deep anesthesia, the left sciatic nerves were removed for gas chromatography and histology. Nerve segments were weighed both wet and dry to calculate water content:

\[
\text{Water content} = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}}
\]

Gas chromatographic measurements of nerve sugars and polyols were made with \(\alpha\)-methylmannoside as an internal standard using a Hewlett-Packard gas chromatograph (Hewlett-Packard 5890; Avondale, PA) fitted with a flame ionization detector and a 25 m \(\times\) 0.2 mm capillary column (Hewlett-Packard Ultra 1) as described previously (Calcutt et al., 1994b).

For histology and morphometry, sciatic nerves were fixed by immersion for at least 24 h in cold 2.5% phosphate-buffered glutaraldehyde. Following standard osmication, dehydration, and embedding in araldite for light and electron microscopy, transverse 1 mm sections were cut from a block containing nerve sampled midway between the sciatic notch and the popliteal fossa. The total number of endoneurial vessels per nerve was counted by light microscopy in sections stained with \(p\)-phenylenediamine.

Electron microscopic examination was conducted using ultrathin sections of the entire tibial (largest) fascicle of each sciatic nerve. A negative was obtained for every endoneurial vessel that contained five or more granules or vacuoles which together occupied at least 50% of the cytoplasm. Five or more granules or vacuoles was arbitrarily selected as a minimum criterion to insure unequivocal recognition of mast cells. Mast cell profiles with fewer than five granules or vacuoles were not counted; however, these profiles were rare and equally distributed over all groups. Each mast cell was classified as degranulated (if at least two granules were decreased in density or at least one granule was fusing with the membrane or showed reduction in density compared to others), or intact.

To the fullest possible extent, all experiments and data collection were conducted with animals and tissue coded to minimize experimenter bias. All data were included in the final analysis except where the quality of the material was insufficient for morphologic measurements due to mechanical artifact or poor fixation. Screening of tissue was performed without knowledge of group identity and resulted in the exclusion of two samples from each of the control and ARI-treated galactose groups and one from the untreated galactose group. The possibility of differences occurring between the control, untreated galactose and ARI-treated galactose groups was tested by one-way ANOVA. If a statistically significant difference was found, then multiple comparisons were made using the Student-Newman-Keuls procedure. However, if significant differences between group variances were detected by Bartlett's test, data were analyzed by Kruskal-Wallis nonparametric
Table 1

<table>
<thead>
<tr>
<th>Water content (mg/mg dry wt)</th>
<th>Galactose and polyol content (nmol/mg dry wt)</th>
<th>Galactose</th>
<th>Dulcitol</th>
<th>myo-Inositol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>12.55 ± 1.78</td>
</tr>
<tr>
<td>Galactose</td>
<td></td>
<td>1.50 ± 0.24</td>
<td>ND</td>
<td>12.55 ± 1.78</td>
</tr>
<tr>
<td>Galactose + ARI</td>
<td></td>
<td>1.98 ± 0.29</td>
<td>8.80 ± 8.62</td>
<td>5.26 ± 0.57</td>
</tr>
<tr>
<td>Kruskal-Wallis</td>
<td></td>
<td>1.36 ± 0.06</td>
<td>8.17 ± 5.78</td>
<td>12.55 ± 1.78</td>
</tr>
<tr>
<td>p vs. b</td>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

AR1 = aldose reductase inhibitor; ND = not detected; NS = not significant. The Galactose group was fed with a diet containing 40% galactose by weight. The AR1-treated group received Tolrestat (50 mg/kg orally by gavage, daily). Data are presented as mean ± SD (n = 6–9 per group). Because of differences between group variances, data for water and myo-inositol content were analyzed by Kruskal-Wallis non-parametric analysis of variance and Dunn’s post-hoc test. Because galactose and dulcitol were not detected in control nerves and in some nerves of the Galactose + AR1 group, the effect of AR1 treatment in galactose-fed rats was tested by an unpaired, two-tailed t-test (galactose content) or, because of differences in variance, by Mann-Whitney U-test (dulcitol content).

analysis of variance and Dunn’s post-hoc test. Unless otherwise stated, all data are presented as mean ± SD.

3. Results

There was no significant difference in initial body weight between control (237 ± 13 g; mean ± SD), galactose-fed (238 ± 15 g) and AR1 treated, galactose-fed animals (234 ± 12 g). However, final body weights were significantly less in AR1-treated, galactose-fed animals (213 ± 20 g) than in control (250 ± 17 g) or galactose-fed (247 ± 18 g) rats. The effects of aldose reductase inhibition on nerve water, galactose, and polyol content are presented in Table 1. Water content was increased in the nerves of galactose-intoxicated rats compared with the control and AR1-treated, galactose-fed groups. Galactose and its metabolite, dulcitol, were detected only in galactose-fed rats. In both groups of galactose-fed rats, nerve galactose levels were similar but the level of dulcitol was significantly reduced in the AR1 treated, galactose-fed group. myo-Inositol levels were significantly decreased in the galactose group compared to controls and AR1-treated, galactose-fed groups.

![Endoneurial mast cells in control and galactose-fed rats. A: Normal-appearing mast cell in control animal. Magnification ×3120. B: Mast cell from a control animal showing a granule which is reduced in density compared to others. Magnification ×4875. C: Degranulating mast cell from Tolrestat-treated, galactose-fed animal. Magnification ×3705. D: A completely degranulated mast cell with numerous empty vacuoles from an untreated, galactose-fed animal. Magnification ×3830. Uranyl acetate and bismuth subnitrate.](image-url)
Table 2
Endoneurial mast cells in cross-section of rat sciatic nerve by electron microscopy after seven days of galactose intoxication

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Intact</th>
<th>Degranulating</th>
<th>Degranulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.8 ± 1.8</td>
<td>1.4 ± 1.5</td>
<td>1.4 ± 1.1</td>
<td>0 ± 2.9</td>
</tr>
<tr>
<td>Galactose</td>
<td>6.9 ± 3.0</td>
<td>2.1 ± 2.0</td>
<td>2.2 ± 1.4</td>
<td>2.6 ± 2.9</td>
</tr>
<tr>
<td>Galactose + ARI</td>
<td>3.9 ± 2.1</td>
<td>1.9 ± 1.4</td>
<td>1.9 ± 2.0</td>
<td>0.1 ± 0.4</td>
</tr>
<tr>
<td>ANOVA/Kruskal-Wallis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a vs. b</td>
<td>p &lt; 0.005</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; 0.005</td>
</tr>
</tbody>
</table>

Table 2
Endoneurial mast cells in cross-section of rat sciatic nerve by electron microscopy after seven days of galactose intoxication

By electron microscopy, the total number of mast cells per sciatic nerve tibial fascicle was significantly greater in the galactose-fed group than in the control or ARI-treated, galactose-fed groups (Table 2). Although no difference was seen in the numbers of intact or degranulating mast cells between any group, an increase in degranulated mast cells was found in galactose-fed animals compared to both control and ARI-treated, galactose-fed animals. Examples of intact, degranulating, and degranulated mast cells are shown in Fig. 1.

The number of vessels counted by light microscopy was not significantly different between control (47 ± 4; mean ± SD), galactose-fed (51 ± 6) and ARI-treated, galactose-fed animals (45 ± 3). The number of vessels counted by electron microscopy was less than the total number observed by light microscopy. However, electron microscopic vessel counts were not significantly different between control (17 ± 3; mean ± SD), galactose (20 ± 4) and ARI-treated, galactose (17 ± 3) groups.

Examples of vessels viewed by electron microscopy are shown in Fig. 2. Some vessels in galactose-fed animals showed partial constriction of the lumen (Fig. 2C). Profiles were seen in which the lumen was completely occluded by monocytes (Fig. 2D), although these were rare and equally distributed across all groups (control, 3 occluded profiles per 126 vessels examined; untreated galactose, 1/156; and ARI-treated galactose, 1/133). Constricted or occluded profiles contrast with the patent vessels observed in control (Fig. 2A) and ARI treated, galactose-fed animals (Fig. 2B). Measures relevant to vessel constriction, including luminal and outer wall perimeters and areas, revealed a statistically significant narrowing of vessels from nerves of galactose-fed rats compared to either control or ARI-treated, galactose-fed groups (Table 3). By calculation, the vasoconstriction index was significantly greater in the galactose-fed group compared to the control or ARI-treated, galactose-fed groups (Table 3).

Table 3
Measurements by electron microscopy and stereology of average endoneurial vessel perimeters and areas after seven days of galactose intoxication

<table>
<thead>
<tr>
<th></th>
<th>Luminal perimeter (µm)</th>
<th>Outer wall perimeter (µm)</th>
<th>Luminal area (µm²)</th>
<th>Wall area (µm²)</th>
<th>VCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.12 ± 3.0</td>
<td>37.13 ± 5.55</td>
<td>76.17 ± 21.91</td>
<td>61.16 ± 12.85</td>
<td>0.068 ± 0.11</td>
</tr>
<tr>
<td>Galactose</td>
<td>21.13 ± 5.55</td>
<td>32.14 ± 5.92</td>
<td>35.80 ± 21.97</td>
<td>61.45 ± 16.44</td>
<td>0.115 ± 0.04</td>
</tr>
<tr>
<td>Galactose + ARI</td>
<td>28.60 ± 3.0</td>
<td>37.81 ± 3.38</td>
<td>75.09 ± 16.98</td>
<td>68.95 ± 11.35</td>
<td>0.075 ± 0.20</td>
</tr>
<tr>
<td>ANOVA/Kruskal-Wallis</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.001</td>
<td>NS</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>a vs. b</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

AR1 = aldose reductase inhibitor; VCI = vasoconstriction index; NS = not significant. The Galactose group was fed with a diet containing 40% galactose by weight. The ARI-treated group received Tolrestat (50 mg/kg orally by gavage, daily). Data are presented as mean ± SD (n = 8–9 animals per group). Data were analyzed with ANOVA and, if p < 0.05, multiple comparisons were made with the Student-Newman-Keuls test. Because of differences between group variances, data for the VCI were analyzed by Kruskal-Wallis nonparametric analysis of variance and Dunn’s post-hoc test.
4. Discussion

Sciatic nerves of rats fed a diet containing 40% galactose accumulated galactose, dulcitol and water, but were depleted of myo-inositol. As expected, concurrent treatment with Tolrestat by daily oral gavage prevented nerve dulcitol accumulation, indicating efficacy in inhibiting aldose reductase, and restored normal nerve water and myo-inositol levels. Tolrestat treatment also reduced body weight in galactose-fed rats. This effect has previously been noted in Tolrestat-treated control, but not galactose-fed, rats (Calcutt et al., 1994a). However, neither the reduced body weight or the therapeutic effects of Tolrestat are likely to be related to a reduction in food intake, as nerve galactose levels were similar in galactose-fed rats with or without Tolrestat treatment. Thus, this dose of Tolrestat must have other, as yet unknown, metabolic properties that favor weight loss.

In a previous report (Kalichman et al., 1995), endoneurial mast cell numbers were significantly increased after seven days in galactose-fed rats when examined by electron but not light microscopy. It is plausible that the discrepancy between light and electron microscopy is because the increase in the galactose group represents degranulated mast cells, which lack intact granules and are therefore not resolved by metachromatic staining. If this is the case, then previous reports of increased counts of mast cells by light microscopy after longer durations of galactose intoxication (Malmgren et al., 1979; Powell et al., 1981; Forcier et al., 1991) imply that either the incidence of mast cell degranulation is decreased after one or more months of feeding, or that mast cell proliferation increases counts by light microscopy.

Although galactose itself is not known to induce mast cell degranulation the effect of Tolrestat on reducing the number of degranulating mast cells suggests that accumulation of its metabolites may be involved. Aldose reductase catalyzes the conversion of galactose to dulcitol, a polyol that has been implicated in the osmotic disruption of lenticular cells and cataractogenesis (Kinoshita, 1965). In nerve, aside from localization to Schwann cells, avid immunostaining for aldose reductase has been demonstrated in mast cell granules (Powell et al., 1991). Increased flux through this enzyme and accumulation of dulcitol could initiate osmotic disruption of granules and thus induce mast cell degranulation. Inhibition of aldose reductase blocks the conversion of galactose to dulcitol and has been previously shown to prevent the endoneurial osmotic imbalance (Mizisin et al., 1986), increased blood–nerve barrier permeability (Wadhwa et al., 1989) and increased mast cell number (Mizisin and Powell, 1993) in galactose-fed rats. In this quantitative electron microscopic study, mast cell degranulation in galactose-fed rats was also prevented by AR1 treatment.

Endoneurial vasoconstriction in the galactose-fed group was similar to that observed previously after a similar duration of intoxication (Kalichman et al., 1995). A narrowing of vessel lumina is consistent with a reduction in blood flow which has been documented after 6 months of galactose intoxication (Myers and Powell, 1984) but not shorter durations of galactose feeding. The reversal of vasoconstriction by the use of an AR1 has not been previously documented and implies that ARIs might be efficacious in preventing blood flow deficits in galactose neuropathy as has been reported in rats with insulin-deficient diabetes (Calcutt et al., 1994b). This possibility requires further investigation.

The localization of aldose reductase to mast cells (Powell et al., 1991) and to endoneurial endothelial cells (Chakrabarti et al., 1987) is consistent with the prevention of mast cell degranulation and vasoconstriction by Tolrestat in galactose-fed animals. Qualitative observations suggest that mast cell degranulation is prevented by another AR1 after prolonged (4–5 months) galactose feeding (Mizisin and Powell, 1993) and may, as suggested above, be related to preventing polyol accumulation in mast cell granules. Reports of AR1-related effects on vessels include the amelioration of albumin permeability of retinal endothelial cells in galactose-fed rats and diabetic humans (Vinores et al., 1993a,b). In a previous report, closed microvessels were detected in galactose-fed but not diabetic rats (Kalichman et al., 1995) and, in contrast to the microvasculature of nerves from diabetic humans (Sinha et al., 1991), AR1 treatment in the present study prevented vessel closure, as measured by an index of vasoconstriction.

The occurrence of mast cell degranulation and vessel constriction in galactose-fed rats may indicate a causal association. One possibility is that serotonin, a component of mast cell granules (Garrison, 1990), causes endoneurial vasoconstriction by inducing pericyte and smooth muscle contraction. While endoneurial vessels have been reported to have little or no smooth muscle (Bell and Weddell, 1984), smooth muscle and pericytes are present in some vessels as illustrated in Fig. 2. Because many constricted vessels lacked smooth muscle, it is noteworthy that actin-like filaments have been observed in the endothelia of nervous tissue (Le Beaux and Willemot, 1978) and, as contractile elements, may be responsive to serotonin. Contraction of actin-like filaments in endothelial cells and pericytes may explain the vasoconstriction observed in microvessels lacking smooth muscle. However, to our knowledge, the effects of mast cell degranulation on endoneurial vessel morphology are not known.

A combination of endothelial water loss due to osmotic shrinkage induced by endoneurial electrolyte accumulation in addition to increased interstitial pressure could also cause luminal narrowing and microvessel closure in galactose-fed rats (Mizisin et al., 1988). Although there is evidence that aldose reductase is present in endoneurial endothelia in experimental diabetes (Chakrabarti et al., 1987), it is not present in nondiabetic animals (Powell et
al., 1991) nor is it clear when this enzyme is induced in response to hyperglycemia. Given that luminal narrowing is not seen in galactose intoxication of 9–11 months duration (Mizisin and Kalichman, 1993), it is possible that endoneurial vessels are initially unable to protect against water loss by producing osmolytes in response to galactose feeding but acquire this ability later with the induction of aldose reductase. In this context, mast cell degranulation and the subsequent release of the vasodilator, histamine, might be viewed as an inadequate homeostatic attempt to counteract the vasoconstriction and endoneurial osmotic imbalance resulting from seven days of galactose intoxication.

These studies suggest that the endoneurial mast cell degranulation and vasoconstriction seen after seven days of galactose feeding are related to galactose metabolism by aldose reductase. Subsequent studies are required to determine the mechanism by which polyol pathway flux induces mast cell degranulation, whether mast cell degranulation is caused by or is the cause of endoneurial vasoconstriction, and how ARIs prevent increased permeability of the blood–nerve barrier. The possible relationship between mast cell degranulation and vasoconstriction is being tested by treating galactose-fed rats with inhibitors of mast cell degranulation.

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References


