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Inhibition of Kidney Mitochondrial Monoamine Oxidase Activity by the Nephrosis-Producing Aminonucleoside of Puromycin

Paul Bartlett, PhD; Mei-hwa Tu, MS, and Kathleen Ashworth, MS

Intraperitoneal administration in rats of a single large injection of the potent nephrotogenic aminonucleoside, 6-dimethylamino-9-(3'-amino-3'-deoxy-β-D-ribofuranosyl)purine, resulted in striking reductions in kidney cortex mitochondrial monoamine oxidase (MAO) activity, assayed on tyramine substrate. The alterations in enzymatic activity correlated remarkably well with the onset and development of the massive proteinuria characteristic of the disease. The inhibitory effects were also produced in vitro, and Lineweaver-Burk reciprocal plots were clearly indicative of non-competitive inhibition.

It is suggested that aminonucleoside inhibition of kidney mitochondrial MAO activity may be a biochemical lesion in the primary step of GBM collagen cross-link formation, whereby collagen fibrillogenesis is impaired and semipermeability of the GBM to macromolecular plasma proteins altered. Observation of relatively high concentrations of 5-hydroxytryptamine in aminonucleoside-nephrotic rat kidneys suggests significant impairment of serotonin, and probably also catecholamine, metabolism in the diseased kidneys.

Of the many explanations proposed for the semipermeability characteristics and filter-like function of the glomerular basement membrane (GBM), the concept that the membrane has a gel-like structure, in which fine fibrillar-like components are embedded in the amorphous matrix, forming a filtering membrane, seems most acceptable. Such a structure, particularly if it behaves as a thixotropic gel, would permit the passage of particles of all sizes without deformation of the membrane.

Now while fibrillar-like components are seen in fixed sections of GBM subjected to electron microscopy, these probably reflect only a transient state in an ongoing interaction between the collagen-
glycoprotein components in the membrane matrix. At a given point in time one might certainly expect the flux of protein molecules to be affected by a variety of parameters known to influence gel formation. Factors affecting the degree of hydration of the gel, the concentration or ionic strength of electrolytes in the gel — and certainly in the case of GBM collagen — the extent and nature of intramolecular and intermolecular cross-linkages involved in fibrillogenesis must, for example, be considered as affecting, either singularly or in combination, the semipermeability characteristics of such membranes. In this context, the possibility of the existence of a biochemical lesion in the primary step in collagen cross-link formation, whereby collagen fibrillogenesis becomes impaired and semipermeability of the GBM altered, seemed worthy of investigation.

In several tissues, the initial step in the formation of collagen cross-links involves oxidative deamination of the ε-amino group of certain peptide-bound lysyl residues in the collagen polypeptide chains, resulting in the formation of Δ-semialdehydes or allysyl residues. These then spontaneously either cross-link by aldol condensation with another similarly deaminated lysyl residue in another peptide chain, or form an amidine cross-link by Schiff base formation with an ε-amino group of another lysine residue. Studies from several different laboratories on the mechanism of action of the lathyrogenic β-aminopropionitrile (β-APN) and copper deficiency on cross-linking of collagen and elastin have clearly demonstrated that an amine oxidase (E.C.1.4.3.4) is involved in the initial oxidative deamination step.

The present communication specifically relates to monoamine oxidase (MAO) activity in mitochondria isolated from kidney cortex of normal rats and from similar groups of rats treated with the nephrosis-producing aminonucleoside, 6-dimethylamino - 9 - (3'- amino - 3' - deoxy - β - D - ribofuranosyl)purine. Results of ancillary studies relating to the mechanism of action of the aminonucleoside are also reported.

**Methods**

Aminonucleoside nephrosis may be induced in Sprague-Dawley (150-200g) rats either by a single intraperitoneal injection of 15 mg of the compound dissolved in 1 ml of isotonic saline or by daily injection (subcutaneously) of 1.5 mg of the compound per 100 g body weight per day for a period of 10 days. In either case proteinuria commences on or about the fifth to sixth day after administration of the aminonucleoside. Terminally, ie, on the 10th day of the induction period, urinary excretion of protein ranges from 200-595 mg/24 hr collection period.

Both normal and aminonucleoside-treated rats were fasted for 24 hours prior to sacrifice by stunning and exsanguination. The kidneys were immediately excised, decapsulated, and the cortices processed for subcellular fractions as described by Shibko and Tappel. This procedure, although originally designed for the isolation of lysosomal fractions, provides pellets and supernatants which can be further processed for mitochondria, microsomes, and cytoplasmic supernatants.

Monoamine oxidase (MAO) activity was measured by the fluorometric procedure described by Tipton, using tyramine substrate. A standard curve of the production of hydrogen peroxide (expressed in nanomoles, nM) versus fluorescence units (expressed in micromperes) was prepared for each series of assays. Mitochondria equivalent to four different concentrations of mitochondrial protein were used in each MAO assay.

**Preparation of Aminonucleoside**

The aminonucleoside was a gift from the disaccharide aminonucleoside. 6-dimethylamino - 9 - (3'-amino - 3' - deoxy - β - D - ribofuranosyl)purine.

**Results**

Results of the experiments are summarized in Table 1. It is evident from Table 1 that mitochondrial MAO activity is markedly elevated in aminonucleoside-treated rats compared with control animals.
Aminonucleoside-Inhibited Monoamine Oxidase

TABLE 1.

Effects of the Aminonucleoside of Puromycin on Rat Kidney Cortex Mitochondrial Monoamine Oxidase Activity

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Day of experiment*</th>
<th>Urine protein† mg/24 hr</th>
<th>Monoamine oxidase activity‡</th>
<th>Normal rat nM H₂O₂/min/mg protein</th>
<th>Treated rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4</td>
<td>35 (17-52)</td>
<td>1.00</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>123 (83-167)</td>
<td>0.97</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>359 (300-425)</td>
<td>1.10</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>398 (131-680)</td>
<td>0.86</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>10</td>
<td>398 (131-680)</td>
<td>1.02</td>
<td>0.57</td>
<td></td>
</tr>
</tbody>
</table>

In vitro Experiment

| VI a. Normal Assay | 1.10 |
|                   |      |
| b. Same as a plus aminonucleoside (1.35 x 10⁻⁴ M) | 0.81 |

*Days after injection of treated rats with a single 15 mg dose of aminonucleoside dissolved in 1 ml isotonic saline.
†Mean and range of excretion for rats in treated group. Normal rats averaged 7 mg/24 hr; with a range of 2-24 mg/24 hr.
‡Monoamine oxidase activity determined as described by Tipton, in mitochondria isolated from the pooled kidney cortex of 3 rats per experiment.

Results and Discussion

The sixth experiment was an in vitro one, in which mitochondria from normal rat kidney cortex were incubated in the absence of aminonucleoside and with 1.36 x 10⁻⁴ M aminonucleoside added during the 10 minute assay period. Presence of the aminonucleoside produced a striking inhibition of MAO activity (about 26%).
Some insight into the mechanism of the inhibitory effects of aminonucleoside on kidney mitochondrial MAO activity is provided by the Lineweaver-Burk reciprocal plot shown in Figure 1. In the presence and absence of $2 \times 10^{-4}$ M aminonucleoside, the $1/V$ versus $1/S$ plots intersect on the baseline—clearly indicating a purely non-competitive inhibition. From the baseline intersection, a $K_M$ value of $1.65 \times 10^{-4}$ M was calculated. From the points of intersection of the reciprocal plots with the vertical axis and the inhibitor concentration, the $K_i$ value was calculated to be $5.88 \times 10^{-4}$. By the Dixon graphical procedure, a $K_i$ value of $5.35 \times 10^{-4}$ was obtained directly from the plot of $1/V$ versus $i$.

The fact that aminonucleoside has no demonstrable in vitro effect on the peroxidase-stimulated second step of the coupled reaction involved in the Tipton MAO assay, focuses attention on the initial MAO-catalyzed step in which the tyramine substrate is oxidized to an aldehyde, ammonia and hydrogen peroxide. Acting non-competitively, the aminonucleoside does not affect the combination of the substrate with the enzyme, but affects only the velocity of the reaction. While this might be expected in the inhibition of MAO by the substrate tyramine, it is unexpected in the inhibition of MAO by the non-competitive inhibitor aminonucleoside. It is significant that the non-competitive nature of this inhibition correlates with the metabolism of the tyramine substrate to ammonia.
explained by failure of the enzyme-inhibitor-substrate complex to break down (equivalent to an effective reduction in the concentration of active enzyme), it might also be due to actual breakdown of this complex at a velocity less than that of the enzyme substrate, the net effect of which would be a reduction in the measured rate of H$_2$O$_2$ production.

In final analysis, determination of the relevance of the present observations on the inhibitory effects of aminonucleoside on MAO activity in kidney mitochondria to the cross-linking mechanism in GBM collagen, must await conclusive demonstration that the MAO activity assayed on tyramine substrate is indeed the enzymatic activity involved in converting collagen peptide-bound lysyl residues to allysyl residues and that these subsequently, by aldol or aldimine condensation, form the collagen cross-links. Promise of forthcoming purification procedures for an approximately 500-fold purified specific lysyl oxidase enzyme by DEAE-cellulose chromatography should facilitate in-depth studies of the working hypothesis proposed herein.

Now while the foregoing has focused attention on the relationship of inhibited MAO activity to the process of cross-linking and fibrillogenesis in GBM collagen, the relationship of inhibited MAO in tubule cell mitochondria to altered cross-linking and fibrillogenesis in proximal tubule cell basement membrane collagen should also be examined. Certainly proximal tubule cell mitochondria contribute significantly to the isolated kidney cortex mitochondrial population in which MAO activity is inhibited by aminonucleoside. In this context recently published results of a study of renal tubular effects of the aminonucleoside of puromycin provide some support for the view that a defect in proximal tubular reabsorption of protein may explain the proteinuria of aminonucleoside-treated rats. Doubtful correlation of the time-course of development of such alterations with the initiation and onset of massive proteinuria, however, cautions against acceptance of tubular defects as adequately explaining the pathogenesis of the proteinuria.

However, of even greater significance in the development of the renal disease than the hypothesized effects of aminonucleoside on collagen cross-linking and fibrillogenesis in GBM and tubular cell basement membranes may be the inhibition of the enzymatic activity involved in the oxidative deamination of cellular 5-hydroxytryptamine (serotonin). In this context several observations are of interest. Oliver and coworkers, for example, have reported increased urinary excretion of catecholamines by edematous nephrotic children, and Borges and Bessman have reported a large reduction in the excretion of 5-hydroxyindole acetic acid, a major product of serotonin metabolism, by patients with glomerular nephritis. Accumulation of 5-hydroxytryptamine might thus be expected in kidneys of aminonucleoside-nephrotic rats; and indeed exploratory studies conducted in our laboratory confirm this speculations. Thus, a relatively high concentration of 5-hydroxytryptamine (ie. 2.65±1.09 ngm/g tissue) in the kidneys of rats treated with a series of ten small daily injections of aminonucleoside, in comparison with a value of 1.48±0.21 ngm/g tissue in the kidneys of six normal untreated rats, might be attributed to aminonucleoside-inhibition of MAO activity. Of more than passing interest also are the results of studies on the nephrotoxicity of 5-hydroxytryptamine in the rat. Tubular effects of this substance and those recently reported for the aminonucleoside of puromycin are strikingly similar, ie, necrosis of the proximal convoluted tubule and tubular dilatation. The time-course of occurrence of such changes, ie,
subsequent to the onset of proteinuria, would however seem to place these effects in the category of secondary rather than primary effects of aminonucleoside; and as such they can not be considered as causally related to the initiation and onset of the proteinuria characteristic of the nephrotic syndrome.

Tubular reabsorption of protein might be expected to be reduced in necrotic tubules, however, and may be of sufficient magnitude to contribute significantly to the massive proteinuria of the aminonucleoside-induced nephropathy.

References


