A New Animal Model to Study Endogenous Cardiotonic Steroids and the Progression of Cardiovascular Events in Salt-Sensitive Hypertension

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Abstract

The Dahl salt-sensitive rat is a well-established model to study essential hypertension. We first described a subgroup of these rats based on the unique response pattern in systolic blood pressure during the first weeks of exposure to a high salt diet that included cataract formation. We classified this group as cataract-prone Dahl salt-sensitive rat. We also were able to predict and prevent cataract formation in these rats. Further studies showed an inhibition of lens Na+, K+-ATPase activity which may be in part responsible for the cataract formation. Other studies in Dahl salt-sensitive rats maintained on a high salt diet have also shown decreased Na+, K+-ATPase activity in several tissues and increased levels of endogenous circulating Na, K pump inhibitors. For over 20 years, endogenous cardiotonic steroids have been postulated to inhibit Na+, K+-ATPase in both humans as well as in experimental animal models of hypertension. Recent findings have shown results suggesting that there are several forms of cardiotonic steroids with minor differences in structural functionalities, site of production, and specific pump selectivity. We present original data that supports a role for cardiotonic steroids in disease progression related to increased salt-sensitivity. We found increased levels of free endogenous cardiotonic steroids in those rats that were classified as cataract-prone according to their initial systolic blood pressure response to a high salt intake when compared to non-cataract-prone Dahl salt-sensitive rats and their control Dahl salt-resistant rats. The cataract-prone Dahl salt-sensitive rat is an animal model that can help and contribute to open a new door to possibly elucidate the role of endogenous cardiotonic steroids in the pathogenesis and progression of diseases related to salt-sensitive hypertension.

Keywords

Cardiac glycosides, Cardiovascular diseases, Endogenous cardiotonic steroids, Hypertension, Animal models, Salt-sensitivity, Stroke

Introduction

The Dahl salt-sensitive (DS) rat is a known experimental model of salt-sensitive, volume expansion essential hypertension [1]. We found that approximately 35% of weanling DS maintained on a high salt diet until adulthood had an increased incidence of anterior cortical cataract formation suggesting a possible ion transport defect [2]. The group of rats that developed cataracts were those DS that had an initial higher systolic blood pressure response (SBP) during the first four (4) weeks on a high salt intake. These rats were classified as cataract-prone DS (DSc). Rats that did not conform to the unique SBP response found in DSc were classified as DS unlikely to develop cataracts (DSuc) [2-4]. Intermediate responders were not studied further. Cataractous lesions in the DSc were characterized by marked lenticular and aqueous humor electrolyte imbalance [2]. We then studied the effect of a chronic high sodium diet starting in weanling rats on lenticular ouabain-sensitive Rubidium uptake in DS and Dahl salt-resistant (DR) rats as an index of lenticular Na+, K+-ATPase activity [3]. The decrease in total lens Rubidium uptake in DSc before cataract formation was the result of only decreased ouabain-sensitive uptake suggesting that decreased lenticular Na+, K+-ATPase activity might precede cataract formation.

Cognizant of the different genetic profiles of the rat strains and their subsequent variable response to salt intake, we used Sprague-Dawley (SD) rats from which DS and salt resistant (DR) rats were genetically derived, to characterize active and passive Na+ and K+ transport with the use of the short–circuiting technique in the rat lens during chronic normal NaCl diet [5]. We then studied the effect of normal vs high NaCl chronic intake in the lens of SD weanling rats up to 26-30 weeks of age [6]. Although neither sustained hypertension nor cataract formation was observed in any SD rats, the basal lens electrical parameters (lens short–circuit current, translenticular potential and resistance) were significantly altered by...
high NaCl intake. A similar study was done to evaluate the effect of chronic normal vs high NaCl intake in the lens of DSc, DScn and DR rats [7]. All short circuit current measurements in DSc were done in transparent lenses assessed through slit-lamp microscopy. Although DScn had significantly decreased lenticular values when compared to DR, we found significantly lower levels in DSc when compared to DScn. These data suggest that cataractogenesis in DSc may depend on the degree of salt-sensitivity and that lenticular Na, K-ATPase inhibition may play a pivotal role in the loss of transparency of the lens.

For over 20 years, several forms of endogenous cardiotonic steroids (CTS) have been postulated to inhibit Na, K-ATPase in both humans as well as in experimental animal models of hypertension. We decided to conduct this preliminary study to determine if DS- and DR rats kept on a chronic high salt diet had different levels of endogenous cardiotonic steroids. Endogenous digitals like substances (for the purpose of this study it will be abbreviated DLIF) was one of the first cardiotonic steroids (CTS) to be found in plasma of volume-expanded dogs [8], in newborns, pregnant women and in renal failure [9], human cataractous lenses [10], diabetic women with preeclampsia [11]; it was also proposed to be a determinant of salt restriction [11]. Endogenous CTS encompass various chemically similar compounds that have in common a steroid structure and may inhibit or modulate the activity of the Na, K-ATPase, including ouabain-like substances in hypertension [12], bufodienolide in acute myocardial infarction [14], and telocinobufagenin in terminal renal failure [15]. Although the existence and effects of these endogenous cardiotonic steroids has been extensively studied and discussed [13,16-18], recent published articles reconfirm the complex nature of CTS [19-22].

Taking into consideration our previous findings suggesting a link between degree of salt sensitivity, rapid onset of hypertension and decreased lenticular ionic flow in the cataract-prone DS rats (DSc) [2-7], we measured levels of serum Total and Free DLIF in DS and DR rats kept on a chronic high NaCl intake. The objective of this study was to evaluate if there was a possible link between increased levels of a circulating inhibitor of Na, K-ATPase with the systolic blood pressure (SBP) response and the prediction of cataract formation in the DS rat. The results described in this article are part of studies performed in our laboratory at the Puerto Rico Veteran Administration Medical Center (VAMC), presented and published as an abstract in 1997 [23]. Although we fully recognize the existence of other technology to study the complex nature of CTS, we consider that the use of digoxin polyclonal antibodies has the ability to recognize multiple epitopes on any one antigen [24,25]. Therefore, although not as robust for quantitative CTS experiments, the use of polyclonal antibodies exhibit higher affinity to help amplify the signal from targets with low expression level.

Materials and Methods

A total of 42 weanling female DS and 6 age- and sex-matched DR (both derived from Brookhaven outbred Dahl rats) were studied under conditions previously described [2-7]. Animals were fed a standard chow diet (Purina Mills; 0.4% sodium, 0.65% chloride and tap water). SBP was determined in conscious rats at the age of 4-5 weeks as well as throughout the experiments, using the technique of tap water). SBP was determined in conscious rats at the age of 4-5 weeks as well as throughout the experiments, using the technique of

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### Table 1: Systolic Blood Pressure of rats with a high salt diet intake

<table>
<thead>
<tr>
<th>Type of Rats</th>
<th>Time with high salt intake</th>
<th>Mean (± SD)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSsc (n=6)</td>
<td>0 Weeks (Basal)</td>
<td>108.7 (11.6)</td>
<td>0.13</td>
</tr>
<tr>
<td>DSscn (n=5)</td>
<td>0 Weeks (Basal)</td>
<td>114.5</td>
<td>110.5</td>
</tr>
<tr>
<td>DR (n=6)</td>
<td>0 Weeks (Basal)</td>
<td>89, 119</td>
<td>108, 119</td>
</tr>
<tr>
<td>DSsc (n=6)</td>
<td>1 Week*</td>
<td>140.2 (17.4)</td>
<td>0.37</td>
</tr>
<tr>
<td>DSscn (n=5)</td>
<td>1 Week*</td>
<td>135.0</td>
<td>127.0</td>
</tr>
<tr>
<td>DR (n=6)</td>
<td>1 Week*</td>
<td>122, 170</td>
<td>122, 142</td>
</tr>
<tr>
<td>DSsc (n=6)</td>
<td>2 Weeks</td>
<td>176.5 (17.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DSscn (n=5)</td>
<td>2 Weeks</td>
<td>154.2 (11.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DR (n=6)</td>
<td>2 Weeks</td>
<td>153.0</td>
<td>126.5</td>
</tr>
<tr>
<td>DSsc (n=6)</td>
<td>3 Weeks</td>
<td>184.8 (12.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DSscn (n=5)</td>
<td>3 Weeks</td>
<td>149.4 (15.6)</td>
<td>125.2 (6.8)</td>
</tr>
<tr>
<td>DR (n=6)</td>
<td>3 Weeks</td>
<td>152.0</td>
<td>123.0</td>
</tr>
<tr>
<td>DSsc (n=6)</td>
<td>4 Weeks</td>
<td>181.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DSscn (n=5)</td>
<td>4 Weeks</td>
<td>1516.4 (4.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DR (n=6)</td>
<td>4 Weeks</td>
<td>150.0</td>
<td>127.0</td>
</tr>
<tr>
<td>DSsc (n=6)</td>
<td>7 Weeks</td>
<td>228.5 (14.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DSscn (n=5)</td>
<td>7 Weeks</td>
<td>173.0 (35.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DR (n=6)</td>
<td>7 Weeks</td>
<td>163.0</td>
<td>127.0</td>
</tr>
<tr>
<td>DSsc (n=6)</td>
<td>13 Weeks*</td>
<td>204, 241</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DSscn (n=5)</td>
<td>13 Weeks*</td>
<td>143, 235</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DR (n=6)</td>
<td>13 Weeks*</td>
<td>114, 138</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

**Note:** DSsc: Cataract-prone Dahl Sensitive rats; DScn: Dahl Sensitive unlikely to develop cataract; DR: Dahl Resistant; Woulski-Wallis test was used to determine significance (P<0.05); *One missing value for the DR group; **DScn and DR rats were killed at 7-weeks of receiving a high salt diet; ***DScn rats were killed at 13-weeks of receiving a high salt diet.
the level of SBP, the higher the level of Free DLIF. Such a correlation
was not significantly (P=0.07), by an average of 2.61 pg/ml for every
0.87 pg/ml (P=0.02). Serum Total DLIF in all rats increased, although
in SBP, we found Free DLIF to increase significantly by an average of
22% of the variability in Total DLIF can be explained by measuring the SBP levels. For every additional mm/Hg
in serum Free DLIF and 22% of the variability observed
among the groups (P>0.05). DSc rats were more likely to have higher levels of
differences among the groups. This finding strengthens the suggestion that the significant changes in SBP and,
No significant difference was found using the Kruskal-Wallis test, then a
Mann-Whitney U test was performed using a Bonferroni correction to
to determine which of the groups were significantly different; p-values <0.017 were considered statistically significant. Furthermore, based on results obtained at the moment of death of the rats, a simple linear regression analysis was performed in order to assess the relationship
between SBP (predictor) and levels of DLIF (outcome).

Table 2 shows Total and Free serum DLIF values for each group of rats. Differences in Free DLIF were observed among the three groups (P<0.02). By contrast, Total DLIF did not differ statistically among groups (P=0.05). DSc rats were more likely to have higher levels of serum Free DLIF than DR rats (median values 142 pg/ml vs. 28 pg/ml; P=0.010) and DScnc rats (median value 38 pg/ml).

Figure 1A and 1B shows the association between Free and Total DLIF, respectively, and SBP. About 34% of the variability observed in serum Free DLIF and 22% of the variability in Total DLIF can be explained by measuring the SBP levels. For every additional mm/Hg in SBP, we found Free DLIF to increase significantly by an average of 8.77 pg/ml (P=0.02). Serum Total DLIF in all rats increased, although not significantly (P=0.07), by an average of 2.61 pg/ml for every additional mm/Hg in SBP. It is clear from Figure 1A that the higher the level of SBP, the higher the level of Free DLIF. Such a correlation
did not hold for Total DLIF.

Discussion

Establishing criteria for animal models to be used in cardiovascular disease is a challenging goal due to the complex multifactorial nature of the disease. It has been suggested that an ideal animal model of cardiovascular disease would be one that mimics the human subject metabolically and pathophysiologically, is large enough to permit physiological and metabolic studies, and will develop end-stage disease comparable to those in humans [27]. We agree with the conclusions of Russell JC & Proctor SD [27] that:

- no one species will be suitable for all studies,
- the most useful and valid species/strains for the study of cardiovascular disease appear to be small rodents, rats, and mice,
- this fragmented field would benefit from a consensus on well-characterized appropriate models for the study of different aspects of cardiovascular disease and a renewed emphasis on the biology of underlying diseases

Thus, our rat model is eligible to be used for the study of the relationships among high blood pressure dependent on salt intake, DLIF and cataracts. Varying blood pressure responses to a high salt intake observed in DS has also been demonstrated in clinical studies. When switched from a lower to a higher NaCl diet, increases in salt sensitivity in blood pressure responses were observed in healthy and hypertensive individuals [28-31]. Increased salt-sensitivity has also been shown to be race-related. For example, blacks have a greater frequency of salt sensitivity than whites and a more aggressive hypertension progression leading to increased complications and mortality [28,32-34].

Our results analyzed by linear regression revealed a significant correlation between increased SBP and Free DLIF. No such correlation could be established for Total DLIF, suggesting that the major change in DLIF with increased SBP can be due to a shift of the Bound DLIF (attached to proteins or inactive) fraction to the free (active) DLIF fraction. In addition, it is important to note that serum Total DLIF was increased in all rats in response to the high salt diet with no significant differences among groups. This finding strengthens the suggestion that the significant changes in SBP and, therefore, the consequences of hypertension in this model correlate best with Free circulating DLIF.

With the exception of our studies with DSc and DScnc rats, to our knowledge all published experiments in salt sensitive rats bred from Brookhaven have used the DS rat as a single study group. As a result, minor but significant changes and higher mortality rate in the DSc subgroup in response to a high salt diet might have been overlooked or unnoticed [35]. This is also observed in humans, where approximately 60% of all hypertensive subjects are salt-sensitive and among them there is variability in their salt-sensitivity response to high salt. In the

<table>
<thead>
<tr>
<th>Type of Rat</th>
<th>Serum DLIF pg/ml</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSc (n=6)</td>
<td>1411.8 (314.3)</td>
<td>0.07</td>
</tr>
<tr>
<td>DScnc (n=5)</td>
<td>1602.0 (806.7)</td>
<td></td>
</tr>
<tr>
<td>DR (n=6)</td>
<td>1071.9 (181.4)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Serum DLIF values for each group of rats at the moment of death

Note: DSc: Cataract-prone Dahl Sensitive rats; DScnc: Dahl Sensitive; DS: Dahl Resistant; DSnc: Dahl Resistant 

Figure 1: Association between Serum DLIF values and systolic blood pressure by rat groups. Specifically, figure 1A for Serum Free DLIF and 1B for Serum Total DLIF.

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present study, we found higher levels of Free circulating CTS in DSc before cataract formation when compared to DSnc and DR.

There have been numerous basic and clinical research studies focusing on the interactions between different forms of endogenous CTS and the complications of cardiovascular diseases related to hypertension and salt-sensitive hypertension. Our preliminary results in the DS rats exposed to a high salt diet support the evidence suggesting that CTS may be of crucial importance in the high mortality rate associated with increased blood pressure response, salt sensitivity and renal salt handling [17,36]. Considering that high blood pressure is the number one cardiovascular risk factor for cardiac failure and stroke, [37,38] we propose the DSc rat as an experimental model to study these relationships and further define the role of endogenous cardiotonic steroids as potential early diagnostic biomarkers to identify those individuals who are at higher risk.

In summary, based on extensive studies in our laboratory [2-7] we suggest that the DSc together with DSnc and DR can be used as a new animal model, where the beginning and progression of diseases related to salt-sensitivity and blood pressure can be studied before the onset of pathological findings. It is important for other organ involvement studies in this model that we were able to predict and prevent cataract formation in the DSc rats [39] and showed low plasma renin activity and increased ouabain-sensitive lenticular Rubidium uptake in weanling DSc before the development of sustained hypertension and cataracts [40]. This new model can provide important information that may help elucidate cardiovascular mechanisms or biomarkers for early diagnosis, prevention or treatment of high-risk individuals. The following highlights aim to provide the readers with a quick overview of our core findings:

• DS rats have different salt sensitivities (blood pressure response) when exposed to a high salt diet
• Total DLIF was the same independent of the blood pressure response to high salt
• DS rats with rapid onset of hypertension (DSc) had higher Free DLIF levels

In conclusion, the DSc model may help to expand and strengthen our knowledge of the relationship of salt sensitivity of blood pressure as have been discussed in recent studies and reviews [17,18,36,40-43]. Moreover, the DSc model can help advance knowledge of the pathophysiology of oculary pathology in ageing [44].

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