A Comparison of Nucleoside Transport and Metabolism in Hypertensive and Normotensive Rats

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SUMMARY In a previous study, we discovered that spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) are dissimilar with respect to the depressor potency differentiated between intravenously and intra-arterially infused adenosine. To test the hypothesis that this dissimilarity may reflect a difference between the two strains in adenosine transport or metabolism, we compared the kinetics of nucleoside transport (i.e., \[^{3}H\]uridine uptake) in erythrocytes and the pulmonary disposition of \[^{3}H\]adenosine in SHR versus WKY. \[^{3}H\]Uridine uptake in rat erythrocytes was linear for 4 minutes and inhibitable with dipyridamole. Kinetic analysis (i.e., Hofstee plots) of initial uptake velocity indicated no difference between the two strains with respect to apparent \(K_m\) (196 ± 40 vs 230 ± 29 μM in WKY and SHR, respectively) and maximum velocity (7.5 ± 0.4 vs 8.3 ± 0.5 pmol/2 min/12% Hct in WKY and SHR, respectively). Approximately 50% of \[^{3}H\]adenosine infused into the pulmonary artery of perfused rat lung was transported into the lung, and 85% of this material was incorporated into the nucleotide pool. Radioactivity in the lung perfusate consisted initially of equal amounts of adenosine and inosine; however, within 60 seconds after administration of \[^{3}H\]adenosine most of the effluent radioactivity was inosine. No differences were detected in adenosine uptake, intracellular metabolism, or extracellular metabolism in lung from SHR versus WKY. Our data indicate that any difference between SHR and WKY with respect to the biological response to adenosine cannot be attributed to differences in adenosine disposition and, therefore, must be due to pharmacodynamic differences between the strains.

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KEY WORDS • nucleoside transport • spontaneously hypertensive rats • adenosine • lungs • erythrocytes

In a recent study, we compared the depressor effects of intravenous (i.v.) and intra-aortic (i.a.) infusions of adenosine in spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). As expected, in SHR the depressor potency of adenosine was approximately twice as great with i.a. as compared with i.v. infusions (Figure 1), consistent with the known ability of lungs to transport and metabolize adenosine.2,3 Surprisingly, however, in WKY the depressor potency of adenosine infused i.v. was similar to the potency of adenosine infused i.a., suggesting an apparent lack of adenosine clearance by the lungs of WKY. Given the potential importance of adenosine in the cardiovascular system,4-5 we decided to explore further the biological basis for the lack of differential potency of i.v. versus i.a. infusion of adenosine in WKY.

The similar potency of i.v. and i.a. infusion of adenosine in WKY but not in SHR could be explained by at least three hypotheses. First, it is possible that in SHR pulmonary extraction of adenosine is approximately 50%, whereas in WKY adenosine is not extracted by the lung. Second, it is conceivable that the pulmonary extraction of adenosine is similar in SHR and WKY, but that adenosine releases a vasodilator from the lungs of WKY.
NUCLEOSIDE DISPOSITION IN SHR AND WKY

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SHR (n=6)  WKY (n=7)

FIGURE 1. The effect of adenosine on mean arterial blood pressure in SHR and control WKY. Adenosine was infused either i.v. or into the aortic arch (IAA) in conscious rats. Asterisks indicate a significant difference compared with saline infusion. The p value in the left panel is from analysis of variance. (Adapted from Onishi et al.1 with permission from the American Heart Association).

but not from the lungs of SHR. Therefore, even though adenosine is extracted by the lungs of WKY, the released vasodilator would compensate for the loss of adenosine. Third, it is possible that blood-borne adenosine exerts a hypotensive effect through the central nervous system in WKY but not in SHR. Thus, in WKY, even though adenosine is extracted by the lungs following i.v. infusion, it may circulate to the brain and exert an additional hypotensive effect that makes up for the loss of adenosine across the pulmonary circuit.

In this communication, we report the results of experiments designed to test the first hypothesis. This hypothesis was tested by comparing the kinetics of nucleoside transport in red blood cells taken from WKY and SHR and by comparing the disposition of adenosine in the pulmonary vascular bed of SHR and WKY in situ.

Materials and Methods

Rat Erythrocytes

Male SHR or WKY (Taconic Farms, Germantown, NY, USA) weighing 250 to 300 g were anesthetized with ether. Blood samples, approximately 7 ml, were collected into heparinized tubes by cardiac puncture. Erythrocytes were washed three times by low-speed centrifugation, 200 g for 15 minutes, in 10 ml of cold 0.9% NaCl containing 5 mM Tris HCl (pH 7.4) and 5 mM dextrose. Theuffy coat containing leukocytes and the platelet-rich plasma were discarded. Erythrocytes were resuspended in 0.9% NaCl containing 5 mM Tris HCl (pH 7.4) and 5 mM dextrose to yield a hematocrit of 25 to 30%.

Nucleoside Transport

Washed red blood cells, 300 μl, were added to microfuge tubes containing 300 μl of di-n-butylphthalate. Either dipyridamole (25 μM) or vehicle (12 μl of 50% ME0H/H2O) was added to each tube and preincubated for 3 minutes at 37 °C. [3H]Uridine (0.04 μCi) or [3H]uridine (0.04 μCi) containing different concentrations of unlabeled uridine was added to the appropriate tubes and incubated for the indicated times at 37 °C. The nucleoside carrier in rat erythrocytes transports uridine, yet uridine is not metabolized to any significant extent.6 Nucleoside uptake was terminated by the addition of 500 μl of ice-cold buffer containing an excess of unlabeled uridine followed by rapid centrifugation.3 Blank values were obtained by processing cell samples exposed to [3H]uridine ± dipyridamole at 0 °C. Where applicable, kinetic constants (Km and maximum velocity, Vmax) were determined by linear regression analysis of V versus V/S, Hofstee plot,6 where V is the initial uptake rate and f/S is the extracellular substrate concentration.

Perfusion of Lungs in Situ

Male SHR or WKY (Taconic Farms) weighing 250 to 300 g were given an intraperitoneal injection of pentobarbital sodium (45 mg/kg) and ventilated (tracheostomy) with 95% O2, 5% CO2 using a Harvard (South Natick, MA, USA) small-animal respirator. Cannulation of the pulmonary artery and collection of the perfusate were performed by a slight modification of the procedure of Watkins and Rannels.9 Briefly, a ligature was loosely placed around the pulmonary artery and aorta, and a second ligature was placed under the pulmonary
artery and aorta and rotated under the heart to surround the pulmonary veins and left atrium. A PE-240 cannula with a flanged end was inserted at the base of the left ventricle and gently advanced through the mitral valve directly into the atrial appendage. The ligature around the atria was secured, and the cannula was positioned so that the flanged end of the cannula lay flush at the base of the mitral valve. Immediately following, a small incision was made in the right ventricle, a PE-200 cannula filled with perfusate was slightly advanced into the pulmonary artery, and the ligature was secured. Perfusion was begun at a flow rate of 4 ml/min with a Harvard peristaltic pump, and perfusion pressure was periodically monitored and determined to be between 13 and 18 cm H2O. The total duration of interruption of the pulmonary circulation was less than 5 minutes.

The perfusate was composed of a Krebs-Ringer bicarbonate buffer (pH 7.4) of the following composition: 130 mM NaCl, 2.0 mM CaCl2, 4.6 mM KCl, 1.2 mM MgCl2, 26 mM NaHCO3, 10 mM dextrose, 1.2 mM NaH2PO4, and 4.5% pyrogen-free dextran (molecular weight, 70,000). The perfusate was kept at 37 °C. The first 100 ml of perfusate that passed through the lungs was discarded. [3H]Adenosine (6.0 µCi, in 100 µM unlabeled adenosine) was infused for 10 seconds at a flow rate of approximately 1 ml/min into a catheter directly inserted into the pulmonary artery. We chose 100 µM cold adenosine since this is less than the Km for adenosine that has previously been reported in rat lung.3 Effluent was collected in 15-second fractions for a total of 3 minutes, after which perfusion and ventilation were terminated and the lungs were rapidly dissected free of remaining heart and trachea, frozen at −70 °C in liquid N2, and weighed. Both effluent fractions and lung were stored at −70 °C until analyzed.

**Sample Processing**

Effluent and lung were thawed at room temperature. Duplicate aliquots of effluent were taken from each sample, and total radioactivity was determined using scintillation spectrometry in the presence of 5 ml of Liquiscent (National Diagnostic; Somerville, NJ, USA) scintillation cocktail. A separate 20-µl aliquot was applied to a silica gel thin-layer chromatography plate (Kodak, Rochester, MN, USA) scintillation cocktail. A separate 20-µl aliquot was applied to silica gel thin-layer chromatography plates for chromatographic analysis of adenosine metabolites.

**Thin-Layer Chromatography**

Aliquots of lung effluent (20 µl) and lung extract (25 µl) were applied to plastic-back silica gel plates containing a fluorescent indicator. The plates were developed in a solvent system, which was allowed to equilibrate overnight, composed of n-butanol/ethyl acetate/methanol/ammonia, 7:4:3:4.3 Nucleotides remained at the origin while inosine, hypoxanthine, adenosine, and adenine were resolved with typical Rf values (for 13 cm solvent front height) as follows: inosine, 0.26; hypoxanthine, 0.46; adenosine, 0.57; adenine, 0.73.

Standards were visualized under ultraviolet light, and quenched purine areas were cut out, eluted with 1 ml of distilled water, and transferred to scintillation vials. Then, 15 ml of Liquiscent scintillant was added, and radioactivity was determined by scintillation spectrometry. Approximately 80% of the radioactivity applied was recovered by this procedure.

**Chemicals**

[3H]Uridine (specific radioactivity = 25 Ci/mmol) was obtained from New England Nuclear (Somerville, NJ, USA), and [3H]adenosine (specific radioactivity = 40 Ci/mmol) was obtained from American (Arlington Heights, IL, USA). Dipyridamole, uridine, and adenosine were purchased from Sigma Chemical (St. Louis, MO, USA). Dextran was obtained from U.S. Biochemical (Cleveland, OH, USA). All other chemicals used were reagent grade.

**Results**

Figure 2 illustrates the time course of [3H]uridine uptake at 37 °C by WKY and SHR erythrocytes in the presence and absence of 25 µM dipyridamole. In the absence of dipyridamole, [3H]uridine uptake in erythrocytes from both WKY and SHR was linear and the rates were similar during the first 10 minutes of incubation. Further increases in incubation time resulted in similar maximal [3H]uridine levels in erythrocytes from both WKY and SHR. The initial velocity of uptake was decreased by approximately 50% when either WKY or SHR erythrocytes were preincubated with 25 µM dipyridamole. A concentration of 25 µM was chosen since, in our initial experiments, this concentration provided maximal inhibition of [3H]uridine transport. Uridine uptake (0.6-300 pmol) into red blood cells.
was found to be saturable and provided linear ($r = 0.96$–0.99) Hofstee plots (Figure 3). Analysis of the data by three independent Hofstee plots revealed no significant differences in either $K_m$ or $V_{max}$ between WKY and SHR red blood cells. However, a 50% decrease in $V_{max}$ and no change in $K_m$ were observed when either SHR or WKY erythrocytes were preincubated with 25 μM dipyridamole (Table 1).

To determine if differences in adenosine uptake or metabolism (or both) exist between WKY and SHR lung, in situ perfusion of both SHR and WKY lungs was performed. [3H]Adenosine, 6.0 μCi, in 100 μM unlabeled adenosine was infused for 10 seconds, and the percent total uptake of [3H]-adenosine in lung and the metabolic fate of [3H]adenosine in lung and lung effluent were determined. Results indicated that total uptake of [3H]adenosine in SHR and WKY lung was similar: 52 ± 3% for WKY and 51 ± 2% for SHR. These results are the mean values ± SEM for six experiments using six separate animals. Analysis for various metabolites of [3H]adenosine by thin-layer chromatography indicated in both SHR and WKY lung that, after 3 minutes of perfusion, more than 85% of [3H]-adenosine taken up by the lung was converted to nucleotides and approximately 10% was converted to the deaminated metabolite inosine. Hypoxanthine and adenosine composed less than 2% of the total radioactivity in both SHR and WKY lung (Figure 4).
TABLE 1. Apparent $K_m$ and $V_{max}$ of $[^3H]Uridine$ Transport in Erythrocytes With and Without Dipyridamole from SHR and WKY

<table>
<thead>
<tr>
<th>Group</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/2 min/12% Hct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>196 ± 40</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>SHR</td>
<td>230 ± 29</td>
<td>8.3 ± 0.5</td>
</tr>
<tr>
<td>WKY + 25 μM dipyridamole</td>
<td>235 ± 21</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>SHR + 25 μM dipyridamole</td>
<td>187 ± 36</td>
<td>3.2 ± 0.7</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM of three separate animals and experiments. Hct = hematocrit.

Red blood cells from WKY and SHR were incubated in duplicate with 0.04 μCi $[^3H]Uridine$ in the presence of increasing concentrations of unlabeled uridine (0.6–300 pmol) for 2 minutes at 37 °C as described in Materials and Methods. Data obtained were subjected to linear regression analysis of $V$ vs $V/[S]$, Hofstee plot, and apparent $K_m$ and $V_{max}$ were determined.

When lung effluent in both SHR and WKY was analyzed by thin-layer chromatography following $[^3H]Adenosine$ infusion, the major radioactive products appearing in the effluent were unchanged adenosine and inosine. Very little hypoxanthine and nucleotides appeared (Figure 5). At early time points both tritiated adenosine and tritiated inosine were significantly represented in the effluent, but after 30 seconds radioactivity was increasing in the inosine fraction and decreasing in the adenosine fraction. By 60 seconds, however, there appeared to be a steady state in terms of release of adenosine (at low levels) and inosine (at high levels). No significant differences were observed in the amounts or in the time course of appearance of the metabolites analyzed between WKY and SHR lung effluent.

**Figure 5. Chromatographic analysis of effluent from control WKY and SHR lungs after infusion of $[^3H]Adenosine$.** WKY and SHR lungs were infused in situ with $[^3H]Adenosine$, and the effluent was collected at 15-second intervals and analyzed for various metabolites of adenosine using thin-layer chromatography as described in Materials and Methods. Results are expressed as the percentage of total radioactivity recovered at specified intervals. Each point represents the mean value ± SEM of six separate animals and experiments.

**Discussion**

The initial observation by Ohnishi et al. that adenosine is more potent in lowering mean arterial blood pressure when given i.a. rather than i.v. in SHR but not in WKY prompted us to investigate the mechanism(s) responsible for this difference. One explanation could be that the extraction of adenosine by the SHR lung is significantly greater than the extraction of adenosine by the WKY lung.

Disposition of adenosine occurs through two mechanisms: 1) facilitated transport into cells followed by intracellular metabolism and 2) extracellular metabolism. To specifically examine the process of adenosine transport in SHR versus WKY, we characterized the kinetic parameters of uridine uptake into erythrocytes. The nucleoside carrier in rat erythrocytes transports uridine, yet uridine is not metabolized to any significant extent by the rat erythrocyte. Therefore, this model of nucleoside transport affords the opportunity to examine exclusively the behavior of the nucleoside carrier without complications caused by adenosine metabolism.
Our data clearly demonstrate that the kinetics of nucleoside transport in the erythrocyte are not different in SHR and WKY, since the apparent $K_m$ and $V_{\text{max}}$ for uridine transport were similar in the two strains. Further, erythrocytes from both strains were equally sensitive to inhibition of transport by dipyridamole. Under our experimental conditions, dipyridamole caused a decrease in $V_{\text{max}}$ without a change in $K_m$. Dipyridamole, a well-characterized blocker of nucleoside transport, has been shown to inhibit nucleoside flux by different mechanisms, depending on the species used in the study. For example, competitive inhibition by dipyridamole has been demonstrated in dog erythrocytes, while inhibition of the mixed type was observed in human erythrocytes. However, these differences in the nature of inhibition by dipyridamole may also be due to differences in the methodology used to determine the kinetic constants.

If the nucleoside carrier on the rat erythrocytes is assumed to be similar to nucleoside carriers in other tissues in the rat, then our data indicate that, in general, adenosine transport is not different in SHR and WKY. However, for at least two reasons, our results in SHR and WKY erythrocytes do not exclude the possibility that pulmonary adenosine clearance is different in SHR and WKY. First, nucleoside transport in SHR and WKY may differ in the lungs but not in the erythrocyte. Second, even if nucleoside transport is similar in SHR and WKY lungs, it is possible that the intracellular or extracellular metabolism of adenosine (or both) is different in SHR and WKY. Therefore, to thoroughly evaluate our hypothesis, it was necessary to compare the net uptake, extraction, and metabolism of adenosine by the lungs of SHR and WKY.

The disposition of adenosine in lungs from SHR and WKY was examined in situ by determining the metabolic fate of [3H]adenosine. These results indicate that the lungs can efficiently transport adenosine out of the vascular compartment and metabolize this captured adenosine into nucleotides. However, our results do not support a difference between SHR and WKY lungs with respect to either the transport or intracellular metabolism of adenosine.

As an index of extracellular metabolism of adenosine, we also examined the profile of [3H]adenosine metabolites appearing in the effluent. Initially, the radioactivity in the lung effluent consisted of approximately equal proportions of adenosine and inosine, with lesser amounts of hypoxanthine and only trace amounts of nucleotides. However, within 30 seconds after the brief infusion of [3H]adenosine, the proportion of radioactivity consisting of adenosine began to decrease concomitantly with a rise in the proportion of radioactivity appearing as inosine. This time profile was similar in SHR and WKY, suggesting that the extracellular metabolism of adenosine by adenosine deaminase did not differ between the two strains.

In summary, our results indicate that the transport, intracellular metabolism, and extracellular metabolism of adenosine are similar in WKY and SHR. Therefore, any differences between the strains with respect to the biological effects of exogenous or endogenous adenosine cannot be attributed to differences in adenosine disposition and must be counted as a real pharmacodynamic difference between the strains.

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References

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