Effects of heparin treatments in vivo and in vitro on adrenal angiotensin II receptors and angiotensin II-induced aldosterone production in rats

Sadahide Azukizawa, Ikuyo Iwasaki, Toshikazu Kigoshi, Kenzo Uchida and Shinpei Morimoto

Division of Endocrinology, Department of Internal Medicine, Kanazawa Medical University, Uchinada, Ishikawa, Japan

Abstract. To evaluate the heparin effects in vivo and in vitro on adrenal angiotensin II receptors and angiotensin II-induced aldosterone production, we examined the angiotensin II binding and the maximum angiotensin II-induced aldosterone production using adrenal glomerulosa cells from rats treated with a heparin preparation containing benzylic alcohol (1500 IU/kg, twice daily for 6 weeks) or cells to which heparin (300 IU/l) was directly added. Comparison was made using the cells from rats treated with vehicle or the cells to which vehicle was directly added. Specific binding of [125I]jodo-angiotensin II was decreased in the cells from heparin-treated rats or in the heparin-treated cells. Scatchard analysis showed that the decrease in binding was due to a decrease in both the number and the affinity of angiotensin II receptors in the cells from heparin-treated rats and a decrease in the number, but not the affinity, of the receptors in the heparin-treated cells. Heparin also caused a decrease in the maximum angiotensin II-induced production, but not the basal production, of aldosterone in the cells from heparin-treated rats and in the heparin-treated cells. These data suggest that heparin interacts with adrenal angiotensin II receptors to inhibit the angiotensin II-induced aldosterone production.

Heparin has been shown to cause a selective aldosterone deficiency with normo- or hyperreninemia in man (Bailey & Ford 1969; Phelps et al. 1980; Leehey et al. 1981; O’Kelly et al. 1983; Sherman & Ruddy 1986) and experimental animals (Uchida et al. 1986). The primary lesion caused by heparin has been thought to be at the adrenal zona glomerulosa because of the lack of renin suppression. In previous studies (Azukizawa et al. 1986; Uchida et al. 1986), we found a selective reduction of the aldosterone response to angiotensin II (AII) in both adrenal zona glomerulosa cells from rats treated with heparin in vivo and the cells to which heparin was directly added in vitro. Since the in vivo heparin treatment also inhibits all the maximum AII-induced production of the aldosterone precursors, pregnenolone, corticosterone and 18-hydroxycorticosterone (Uchida et al. 1986), it is possible that heparin causes an inhibition in adrenal AII receptors rather than a selective impairment of enzyme activity in aldosteronogenesis, leading to a reduction of AII-induced aldosterone production.

To assess this possibility, we examined the effects of heparin treatments in vivo and in vitro on adrenal AII receptors and AII-induced aldosterone production in rats.
Materials and Methods

Male Wistar rats (Std: Wistar/ST strain, Shizuoka Laboratory Animal Centre, Shizuoka, Japan) weighing about 170 g at the start of the present study were used. They were fed a commercial chow (Sanyo Laboratory, Tokyo, Japan) and tap water ad libitum throughout the experiments. To determine the heparin effect in vivo on adrenal glomerulosa cells, the animals were divided into two groups; one was given a heparin preparation containing benzyl alcohol (0.9 v/v %) as preservative (Shimizu Pharmaceutical Co, Tokyo, Japan) im at a dose of 1500 IU/kg body weight twice daily for 6 weeks, and the other received the same volume of vehicle (saline containing 0.9 v/v % benzyl alcohol) for the same period. At the end of the 6th week, these rats were decapitated between 09.00 and 10.00 h, and the adrenals were excised. To evaluate the heparin effect in vitro on the glomerulosa cells, untreated rats at the 6th week of the experiment were decapitated, and the adrenals were excised.

The excised adrenals were bisected and decapsulated, and the capsules were minced. The glomerulosa cells were prepared by the collagenase dispersion technique as previously described (Kim & Morimoto 1979; Uchida et al. 1986). In brief, the minced capsules were pre-incubated in 8 ml of collagenase buffer at 37°C for 50 min under 95% O2—5% CO2. The collagenase buffer consisted of 19.0 µmol/l collagenase (type I) and 0.81 µmol/l deoxyribonuclease in Krebs-Ringer bicarbonate buffer containing 11.1 mmol/l glucose and 4% BSA with a potassium concentration of 3.6 mmol/l (4% BSA-KRBGA). After pre-incubation, the cells were dispersed mechanically and centrifuged at 100 x g for 10 min at room temperature. The supernatant was discarded, and the cell pellet was washed three times with 0.1% BSA-KRBGA. When cell viability was assessed by trypan blue exclusion, the number of viable cells was more than 92% before and after incubation. The fasciculata cell contamination was less than 10%. Since average cell yield on five different occasions (0.4 x 10^5 cells per adrenal) in rats treated with heparin was different from that (1.0 x 10^5 cells per adrenal) in rats treated with vehicle, the washed cell pellet was resuspended in 0.1% BSA-KRBGA to a uniform number of 1 x 10^5 cells/ml per group. For the assay of AII receptors (Douglas et al. 1978), the glomerulosa cells (1 x 10^5 cells/tube) from heparin- or vehicle-treated rats or the cells to which 300 IU/l heparin or vehicle was directly added were incubated in duplicate in a final volume of 1 ml 0.1% BSA-KRBGA with 1.25 x 10^-11 mol/l [125I]monoiodo-AII (SA 1000—1500 µCi/µg) and various amounts of unlabelled AII (0—4.8 x 10^-8 mol/l). After incubation for intervals up to 180 min at 22°C under 95% O2—5% CO2, the receptor-bound and free AII were separated by Millipore filtration using a HART triton-free nitrocellulose filter (0.45 µm, Millipore Corp, Bedford, MA). The filter was washed twice with ice-cold phosphate-buffered saline (pH 7.4), then air-dried, and the bound radioactivity was determined by a gamma spectrophotometer (Auto Well Gamma System, Aloka Co, Tokyo, Japan). All binding is reported as specific binding after subtraction of the non-specific binding observed in the presence of an

![Fig. 1](image_url)

Time course of [125I]monoiodo-AII binding to adrenal glomerulosa cells from rats treated with heparin(1500 IU/kg, twice daily) and vehicle for 6 weeks, or to cells to which heparin (300 IU/l) and vehicle were directly added. Incubations were performed at 22°C in the presence of 1.25 x 10^-11 mol/l [125I]monoiodo-AII. Data are the means ± SEM of 5 experiments. ○—○ cells from vehicle-treated rats; △—△ vehicle-treated cells; ●—● cells from heparin-treated rats; ▲—▲ heparin-treated cells.
excessive unlabelled AII (4.8 × 10^-5 mol/l) from the total binding. Non-specific binding of [125I]iodo-AII to cells plus filters was always less than 0.2% of the total tracer added. For measurement of AII-induced aldosterone production, the glomerulosa cells (1 × 10^5 cells/tube) from heparin- or vehicle-treated rats or the cells to which 300 IU/l heparin or vehicle was directly added, were incubated in duplicate in a final volume of 2 ml 0.1% BSA-KRBGA with or without a maximum aldosterone-stimulating dose of AII (4.8 × 10^-8 mol/l) (Uchida et al. 1986). After incubation for 2 h at 37°C under 95% O2—5% CO2, the cells were sedimented by centrifugation at 1500 × g for 10 min, and the media were stored at -20°C until assayed. Aldosterone in the incubation medium was measured by radioimmunoassay after the separation of aldosterone from cross-reacting steroids by means of high-performance liquid chromatography as previously described (Imaizumi et al. 1987).

The experiments presented here were repeated on five different occasions.

Data are expressed as mean ± SEM. Statistical analysis was performed by Student's paired or unpaired t-test.

Results

Effect of heparin treatments in vivo and in vitro on adrenal AII receptors

The time course of heparin effects in vivo and in vitro on adrenal AII receptors is shown in Fig. 1. The time course of binding of [125I]iodo-AII at 22°C to the glomerulosa cells to which heparin and vehicle were directly added demonstrated that equilibrium was reached after 45 min and remained stable up to 180 min. On the basis of this observation, all incubations for binding studies were carried out for 60 min at 22°C. The binding of AII to adrenal glomerulosa cells from heparin- and vehicle-treated rats in a representative experiment is shown in Fig. 2. In all experiments, Scatchard plots of the binding data were linear. The number and affinity of the receptors in the cells from heparin-treated rats were lower than those in the cells from vehicle-treated rats. Scatchard plots of AII binding to adrenal glomerulosa cells from rats treated with heparin and vehicle in vivo in one of 5 similar experiments. Incubations for the AII binding were performed for 60 min at 22°C in the presence of 1.25 × 10^-11 mol/l [125I]monoiodo-AII. ●—● cells from vehicle-treated rats; ○—○ cells from heparin-treated rats.

Table 1.
Effect of heparin treatment in vivo on adrenal glomerulosa AII receptors.

<table>
<thead>
<tr>
<th></th>
<th>Receptor number (sites/cell)</th>
<th>Receptor affinity (K_a) (× 10^-9 l/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>252 000 ± 40 000^1</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>Heparin</td>
<td>120 000 ± 24 000^2</td>
<td>0.8 ± 0.2^2</td>
</tr>
</tbody>
</table>

Receptor number and affinity calculated by Scatchard analysis. The receptor number (maximum AII binding capacity) are expressed as the number of receptor sites per cell. K_a = equilibrium association constant.

^1 Data are means ± SEM of values obtained from 5 experiments.

^2 P < 0.05 vs corresponding value with vehicle.
than those in the cells from vehicle-treated rats ($P < 0.05$) (Table 1). Typical Scatchard plots of the binding of AII to the cells to which heparin and vehicle were directly added are shown in Fig. 3. In all experiments, the receptors were associated with a single class of binding sites. Scatchard analysis of the data showed a decrease in the receptor number in the heparin-treated cells compared with the vehicle-treated cells ($P < 0.05$). The receptor affinity was similar in both groups (Table 2).

**Effect of heparin treatments in vivo and in vitro on aldosterone production**

Heparin effects in vivo and in vitro on the maximum AII-induced aldosterone production are shown in Table 3. Basal aldosterone production, when corrected to a uniform number of cells per group, was similar in the cells from heparin-treated and vehicle-treated rats and in the cells to which heparin and vehicle were directly added. The maximum AII-induced aldosterone production in the cells from heparin-treated rats and in the heparin-treated cells was lower than that in the cells from vehicle-treated rats and in the vehicle-treated cells, respectively ($P < 0.001$).

**Discussion**

Since collagenase-dispersed rat adrenal cells have been shown to be a useful tool in comparing the binding characteristics of AII receptors with cellular responses of aldosterone production (Douglas et al. 1978), the heparin effects in vivo and in vitro on adrenal AII receptors and AII-induced aldosterone production were examined in the present study using isolated rat adrenal glomerulosa cells. The heparin doses used in the present in vivo and in vitro experiments were those (1500 IU/kg and 300 IU/l, respectively) used in previous studies (Uchida et al. 1986; Azukizawa et al. 1988).

**Table 2.**

<table>
<thead>
<tr>
<th>Receptor number (sites/cell)</th>
<th>Receptor affinity ($K_a$) ($\times 10^{-9}$ l/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>258 000 ± 43 000</td>
</tr>
<tr>
<td>Heparin</td>
<td>168 000 ± 27 000</td>
</tr>
</tbody>
</table>

Receptor number and affinity calculated by Scatchard analysis. The receptor number (maximum AII binding capacity) are expressed as the number of receptor sites per cell. $K_a$ = equilibrium association constant.

1 Date are means ± SEM of values obtained from 5 experiments.
2 $P < 0.05$ vs corresponding value with vehicle.
The results of the present study demonstrate that heparin inhibits the AII binding to adrenal glomerulosa cells in vivo and in vitro. In the cells from rat treated with heparin in vivo, both the number and the affinity of AII receptors decreased. The cells to which heparin was directly added in vitro, however, showed a decrease in the number, but not in the affinity, of AII receptors.

The mechanism for the observed difference between the heparin effects in vivo and in vitro is not clear. It may be related to the duration of exposure to heparin. Prolonged exposure to heparin in vivo could alter not only the number but also the affinity of AII receptors directly or by forming some active substance, in addition to a heparin-induced decrease in the mass of the glomerulosa tissue (Vallent et al. 1964; Wilson & Goetz 1964).

Heparin also caused a decrease in the maximum AII-induced production, but not the basal production, of aldosterone in the cells from heparin-treated rats and in the cells to which heparin was directly added. No data on the heparin effect in vitro on the production of the precursor steroids of aldosterone are available. In our previous report (Uchida et al. 1986), however, the glomerulosa cells from rats treated with heparin in vivo exhibited a decrease in the maximum AII-induced production, but not the basal production, of pregnenolone, corticosterone, 18-hydroxy cortisol, and aldosterone, suggesting that the site of the inhibitory action of heparin is prior to the early step of the aldosterone biosynthetic pathway. Thus, the present inhibitory effects of heparin in vivo and in vitro on both adrenal AII receptors and AII-induced aldosterone production, together with our previous data, suggest that heparin interacts with adrenal AII receptors to inhibit the AII-induced aldosterone production.

In rats, AII is well known to up-regulate its own receptors in adrenal glomerulosa cells in contrast to
to the down-regulation seen in vascular smooth muscle receptors (Douglas & Brown 1982). The in vivo heparin treatment caused a decrease in both adrenal AII receptors and AII-induced aldosterone production despite a known increase in circulating AII (Uchida et al. 1986), leading to an interruption of a regular relation between AII and its adrenal receptors. Such changes, however, seem reversible, since the heparin-induced aldosterone deficiency in man disappears rapidly after the withdrawal of the drug (Phelps et al. 1980; Leehey et al. 1981; O’Kelly et al. 1983). To clarify the reversion of the decrease in adrenal AII receptors after the cessation of heparin in rats, further studies will be required.

References

Received May 9th, 1988.
Accepted July 4th, 1988.

Dr Shinpei Morimoto,
Division of Endocrinology,
Department of Internal Medicine,
Kanazawa Medical University,
Uchinada,
Ishikawa 920-02,
Japan.