AT₁ and AT₂ Receptor Blockade and Epinephrine Release During Insulin-Induced Hypoglycemia

René H. Worck, Erik Frandsen, Hans Ibsen, Jørgen Søberg Petersen

Abstract—Angiotensin II facilitates epinephrine release during insulin-induced hypoglycemia, and this effect appears to be independent of type 1 angiotensin II (AT₁) receptors in man. In the present study, we hypothesized that the action of angiotensin II on adrenomedullary epinephrine release is mediated by an AT₂ receptor-dependent mechanism. In conscious chronically instrumented rats, we measured plasma concentrations of catecholamines during acute insulin-induced hypoglycemia in groups of rats pretreated with the AT₁ receptor antagonist losartan (10 mg/kg IV), the AT₂ receptor antagonist PD123319 (30 mg/kg IV), combined losartan + PD123319, the converting enzyme inhibitor enalapril (1 mg/kg IV), or vehicle. In vehicle-treated rats, the area under the curve for changes in plasma epinephrine concentration was 111 ± 8 nmol·h/L (±SEM). Pretreatment with losartan alone did not affect AUC(plasma epinephrine) (113 ± 17 nmol·h/L), while pretreatment with PD123319 tended to reduce the response (87 ± 10 nmol·h/L, P = 0.8 versus vehicle). However, AUC(plasma epinephrine) was significantly reduced in rats that were pretreated with combined losartan + PD123319 (68 ± 5 nmol·h/L, P < 0.01 versus vehicle) or enalapril 86 ± 10 nmol·h/L (P < 0.05 versus vehicle). Thus, combined treatment with losartan + PD 123319 proved more effective in attenuating the reflex increase in plasma epinephrine concentration during hypoglycemia than either of the two AT receptor antagonists given alone. We speculate that angiotensin II through binding to both receptor subtypes facilitates the sympathoadrenal reflex response by actions at several anatomical levels of the neural pathways involved in the sympathoadrenal reflex response elicited during insulin-induced hypoglycemia (Hypertension. 2001;31[part 2]:384-390.)

Key Words: hypoglycemia ▪ angiotensin receptor subtypes ▪ epinephrine ▪ catecholamines

Insulin-induced hypoglycemia is a powerful stimulus for sympathetic activation. However, in contrast with other sympathoexcitatory stimuli such as hemorrhage, exercise, or environmental stress, severe hypoglycemia produces a relatively selective stimulation of the adrenal medulla. Thus, in humans, severe hypoglycemia causes a 50-fold increase in the plasma concentration of epinephrine while the plasma concentration of norepinephrine only increases about 3-fold. The increased level of epinephrine is considered one of the most important counterregulatory hormones released during severe hypoglycemia, and therefore, any substance that impairs the release of epinephrine may potentially provoke episodes of hypoglycemia in patients with insulin-dependent diabetes mellitus.

Angiotensin II interacts with the sympathetic nervous system at several levels. It stimulates central sympathetic outflow and neurotransmission in sympathetic ganglia, and it facilitates norepinephrine release from sympathetic nerve endings. Furthermore, several lines of evidence suggest that angiotensin II may also stimulate adrenomedullary epinephrine release. High concentrations of angiotensin II stimulate epinephrine release in the isolated perfused rat adrenal gland. In anesthetized cats, it was reported that nonselective angiotensin II receptor blockade with Sar-Ile-Ang II abolished adrenomedullary catecholamine release during insulin-induced hypoglycemia as well as during hemorrhage. Similar findings were reported in anesthetized rats, where the increase in plasma epinephrine concentration observed during hypoglycemia was reduced by CEI.

To examine the role of the renin-angiotensin system for adrenomedullary epinephrine release during insulin-induced hypoglycemia in humans, we studied the effect of CEI with enalapril and the effect of AT₁ receptor blockade with losartan in healthy subjects. Whereas captopril attenuated epinephrine release during hypoglycemia, losartan did not affect the plasma epinephrine concentration either during rest or during hypoglycemia. These findings are consistent with the hypothesis that the AT₂ receptor may be involved in the action of angiotensin II on adrenomedullary epinephrine release in humans. This notion is supported by recent studies which have demonstrated that the AT₂ receptor is the predominant angiotensin II receptor in both human and rat adrenal medulla.
Furthermore, the AT₂ receptor is present in areas of the central nervous system that are involved in sensing neuroglycopenia and in the central regulation of sympathetic outflow. This suggests that withdrawal of AT₂ receptor stimulation may impair adrenomedullary epinephrine release during hypoglycemia both at the level of the adrenal medulla and at the level of the central nervous system.

The aim of this study was to systematically examine the effect of selective pharmacological blockade of AT₁ and AT₂ receptors on the adrenomedullary epinephrine release elicited during insulin-induced hypoglycemia. Experiments were performed in chronically instrumented, conscious rats that were not confounded by acute surgery, environmental stress, drugs, or maneuvers which could affect adrenomedullary epinephrine release to compare the effect of selective angiotensin II receptor blockade with that of CEI, experiments were performed in groups of rats pretreated with either selective AT₁ or AT₂ receptor blockade, combined AT₁ + AT₂ receptor blockade, or CEI by enalapril.

**Methods**

**Materials**

Barter-bred and specific pathogen-free male Sprague-Dawley rats (235 to 380 g) were purchased from Møllergården, Lille Skensved, Denmark. Rats were housed individually at the Panum Institute animal care facility in rooms with constant humidity, temperature, and a 12-hour light/dark cycle. Rats were given free access to tap water and a commercial standard rat diet (Altromin catalog no. C 1314, Altromin International). Experiments were performed in compliance with the ethical code for laboratory animal care issued by the Danish Ministry of Justice.

**Surgery**

Surgical procedures were performed during halothane anesthesia (2% vol/vol) in a 2:1 mixture of N₂O and O₂. Permanent Tygon catheters were inserted into the abdominal aorta and into the inferior caval vein using aseptic techniques. Catheters were tunneled subcutaneously to the back of the neck and exteriorized and fixed as described previously. Catheters were filled with a solution containing 50% glucose added to 1000 IU heparin/ml and 7500 IU streptokinase/ml. After instrumentation, animals were allowed at least 7 days of recovery to habituate the rats to restraining, rats were placed in an experimental restraining cage for several hours during the day before the experiment.

**Experimental Protocol**

Rats were fasted overnight before the experiment. All experiments were performed in a quiet laboratory environment, and care was taken to avoid environmental stress during the experiment. The rat was placed in a restraining cage and allowed an equilibration period of at least 30 minutes duration before a reference blood sample was drawn (t=0) to determine plasma glucose, plasma epinephrine, plasma norepinephrine, and PRA before drug administration. After the first reference blood sample was collected, rats were randomly assigned to pretreatment with either vehicle (isotonic saline, 1 mL/kg IV, n=11), pretreatment with either vehicle (isotonic saline, 1 mL/kg IV, n=11), or pretreatment with either selective AT₁ or AT₂ receptor blockade, combined AT₁ + AT₂ receptor blockade, or CEI by enalapril.

The AT₁ receptor antagonist losartan (10 mg/kg IV, n=8), the AT₁ receptor antagonist PD123319 (30 mg/kg IV, n=10), combined AT₁ and AT₂ receptor blockade (losartan, 10 mg/kg IV + PD123319, 30 mg/kg IV, n=8), or CEI with enalapril (1 mg/kg IV, n=11). The efficacy of AT₁ receptor blockade was evaluated by measuring the blood pressure response to an IV injection of angiotensin II (1 μg), and the efficacy of CEI was evaluated by studying the blood pressure response to IV administration of angiotensin I (100 pg). The efficacy of pharmacological blockade was evaluated before pharmacological blockade, 15 minutes after pharmacological blockade, and at the end of the experiment. Thirty minutes after pharmacological blockade a second reference blood sample (t=0) was drawn. Then hypoglycemia was induced by insulin, 4 IU/kg IV, and arterial blood samples were collected every 15 minutes for the following 75 minutes. After 65 minutes, the plasma glucose level was normalized by IV administration of glucose (2.4 mmol/kg). Blood samples (300 μl each) were drawn into precooled test tubes containing 1 mg of EGTA and 0.7 mg of reduced glutathione and kept on ice during handling. The blood was centrifuged at 4°C for 15 minutes, and plasma was stored at −20°C until analysis. All blood samples were immediately replaced by IV infusion of fresh donor blood obtained from littermates, which were pair-fasted overnight. At the end of the experiment, the rat was killed with an IV overdose of pentobarbital.

**Blood Pressure and HR Recording**

MAP was measured continuously with a pressure transducer (Statham P23 XL) and displayed on a Grass model 7D polygraph (Grass Instruments HR) was recorded by a linear photodetector (Grass model 7P4) triggered by the arterial pressure waveform. Analog signals

| TABLE 1. Efficacy and Selectivity of Renin-Angiotensin System Blockade |
|-----------------|-----------------|-----------------|-----------------|
| Antagonist      | Losartan        | PD123319        | PD123319/Enalapril |
| Δ MAP 1 (mm Hg) | Δ MAP 2 (mm Hg) | Δ MAP 3 (mm Hg) | Δ MAP 4 (mm Hg)  |
| Ang II          | Ang II          | Ang II          | Ang II          |
| 56 ± 4          | 56 ± 4          | 56 ± 4          | 56 ± 4          |
| Ang II          | Ang II          | Ang II          | Ang II          |
| 56 ± 4          | 56 ± 4          | 56 ± 4          | 56 ± 4          |

The effect of selective angiotensin receptor blockade and converting enzyme inhibition on the blood pressure response to intravenous bolus injections of 1 μg of angiotensin II (Ang II) or 100 pg of angiotensin I (Ang I) before (Δ MAP 1), 15 minutes after injection of blocker/inhibitor (Δ MAP 2), and at the end of the experiment (Δ MAP 3). Values are mean±SEM.

*P< 0.05 vs vehicle |
**P< 0.01 vs vehicle |
**P< 0.001 vs vehicle |
*P< 0.05 vs baseline |
**P< 0.01 vs baseline |

**TABLE 2. PRA**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>00 min</th>
<th>0 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.43±0.08</td>
<td>0.35±0.03</td>
<td></td>
</tr>
<tr>
<td>Losartan</td>
<td>0.33±0.06</td>
<td>11.92±1.92</td>
<td>3.94±0.86</td>
</tr>
<tr>
<td>PD 123319</td>
<td>0.37±0.04</td>
<td>0.36±0.09</td>
<td>0.36±0.03</td>
</tr>
<tr>
<td>Losartan+PD 123319</td>
<td>0.49±0.04</td>
<td>15.41±2.82</td>
<td>3.04±0.46</td>
</tr>
<tr>
<td>Enalapril</td>
<td>0.44±0.03</td>
<td>4.60±1.06</td>
<td>1.74±0.46</td>
</tr>
</tbody>
</table>

PRA (ng Angiotensin I/50 μl×3 hours) at baseline (t=00) and after selective angiotensin receptor blockade and converting enzyme inhibition (t=0) and during hypoglycemia (t=60). Values are mean±SEM.
TABLE 3. MAP and HR before Hypoglycemia

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Vehicle</th>
<th>Losartan</th>
<th>PD123319</th>
<th>Losartan+PD</th>
<th>Enalapril</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mm Hg)</td>
<td>116±3</td>
<td>104±6</td>
<td>113±3</td>
<td>96±4*</td>
<td>103±2†</td>
</tr>
<tr>
<td>HR (beats per minute)</td>
<td>382±8</td>
<td>441±15*</td>
<td>384±12</td>
<td>448±13†</td>
<td>423±13*</td>
</tr>
</tbody>
</table>

Values are mean±SEM

*P< 0.05 vs vehicle
†P< 0.01 vs vehicle

were digitized using an AT-MIO-16XE-50 board (National Instruments) and sampled at 1000 Hz using a data acquisition program written in LabView (National Instruments) and developed at the Department of Pharmacology, University of Copenhagen, in collaboration with Bør Data.

Biochemical Analyses

Plasma glucose concentrations were determined on a model 6517 Glucose Analyzer II (Beckman Instruments). Plasma catecholamine concentrations were determined using high-pressure liquid chromatographic separation of radioenzymatically labeled catecholamines as described in detail previously. Intra-assay and interassay coefficients of variation for epinephrine were 4.2% and 12.2%, and those for norepinephrine were 3.8% and 8.5% respectively. PRA was determined using a modification of the antibody trapping procedure originally described by Poulsen and Jørgensen. All analyses were done in duplicate.

Drugs

Insulin (Novo Nordisk), 4 IU/ml, was dissolved in isotonic saline added 1% human serum albumin and stored at 5°C. Losartan (DuPont Pharmaceuticals Inc) and enalapril (Sigma Chemical Co) were dissolved in isotonic saline to final concentrations of 10 and 1 mg/ml, respectively. PD123319 was a generous gift from Parke-Davis Pharmaceutical Research. PD123319 was dissolved in isotonic saline to a concentration of 300 μg/ml and 2 μg/ml. All drugs except insulin were stored at −20°C until use.

Data Analysis

To relate plasma concentrations of glucose, epinephrine, and norepinephrine to MAP and HR at the time when blood was sampled, the

Results

Efficacy of Pharmacological Blockade

AT₁ receptor blockade with losartan inhibited the pressor response to exogenous angiotensin II effectively with no significant changes in the degree of blockade during the experiment (P=16) (Table 1). Combined AT₁ and AT₂ receptor blockade abolished the response to angiotensin II, and the pressor response was significantly less than that observed during AT₁ receptor blockade alone (P< 0.05). AT₂ receptor blockade alone did not affect the pressor response to angiotensin II. Immediately after CEI, the pressor response to angiotensin I was attenuated by about 88%. However, CEI was less effective at the end of the experiment than immediately after administration, as evidenced by an increased pressor

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Time course evolution of plasma glucose before and after insulin and glucose injection. *P< 0.05 for mmol/L decrease of glucose from t=0 to t=15 for PD123319 vs vehicle. Values are mean±SEM.

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** AUC for plasma glucose during hypoglycemia after pretreatment indicated *P< 0.01 vs vehicle †P< 0.01 for AUC vs vehicle. Values are mean±SEM.
response to IV angiotensin I (P<01) The effect of pharmacological blockade on circulating rennin activity (PRA) is shown in Table 2 Blockade of the AT₁ receptor-mediated feedback inhibition of rennin release evoked a marked increase in PRA during AT₁ or combined AT₁ and AT₂ receptor blockade while CEI only caused a moderate increase in PRA

**Effect of Pharmacological Blockade on Insulin-Induced Hypoglycemia**

IV administration of insulin, 4 IU/kg, produced a rapid and sustained hypoglycemic response in all insulin-treated groups (Fig 1) Plasma glucose was significantly decreased already at 15 minutes after insulin administration In insulin-treated groups, plasma glucose stabilized around 1 6 mmol/L, and it was rapidly reversed by an IV bolus injection of glucose (2 8 mmol/kg)

At IC for vehicle-treated rats tended to be slightly smaller than AUC for plasma glucose in the intervention groups, however, this difference was only significant for vehicle versus PD 123319 (6 17±0 25 mmolXh/L versus 7 72±0 54 mmolXh/L, P<01) (Fig 2)

**Effect of Insulin-Induced Hypoglycemia on Plasma Epinephrine**

The effects of insulin-induced hypoglycemia on the plasma concentration of epinephrine and the corresponding AUC are shown in Figs 3 and 4 During insulin-induced hypoglycemia, plasma epinephrine increased about 50-fold in vehicle-treated rats (Fig 3) Losartan alone did not affect the overall epinephrine response during hypoglycemia (Fig 4) However, pretreatment with the combination losartan + PD 123319 caused a significant attenuation of the overall epinephrine response (AUC 68±5 versus 111±8 nmolXh/L, P<001) Enalapril also attenuated the overall epinephrine response (AUC 86±10 versus 111±8 nmolXh/L, P<05) PD 123319 tended to reduce the overall response (87±10 nmolXh/L), but the difference relative to the response in vehicle-treated rats did not reach the level of statistical significance (P=08) Furthermore, the overall epinephrine release was further decreased in rats treated with the combination of losartan + PD 123319 (68±5 mmolXh/L), and this response was significantly different from responses observed in rats pretreated with vehicle (P<001) or losartan (P<05) and tended to be lower than responses after blockade with PD 123319 (P=059) (Fig 4)

**Effect of Insulin-Induced Hypoglycemia on Plasma Norepinephrine**

The effects of insulin-induced hypoglycemia on the plasma concentration of norepinephrine and the corresponding AUC are shown in Figs 5 and 6 During insulin-induced hypoglycemia, plasma norepinephrine increased about 3-fold in vehicle-treated rats (Fig 5) The overall norepinephrine responses were similar among insulin-treated groups although losartan tended to potentiate this response (P=08), however, AUC was significantly decreased in rats treated with the combination of PD 123319 + losartan compared with losartan alone (7 7±0 5 versus 15 6±2 6 nmolXh/L, P<01)

**Effect of Pharmacological Blockade on Blood Pressure and HR During Insulin-Induced Hypoglycemia**

Losartan alone and PD 123319 + losartan caused a significant reduction in MAP (P<05 for both), while enalapril and PD 123319 did not affect baseline MAP Insulin-induced hypoglycemia elicited a marked and similar pressor response in all groups Pretreatment with losartan, losartan + PD 123319, and enalapril produced a significant increase in baseline HR (P<01 for all three groups) Insulin-induced hypoglycemia was associated with a significant and similar bradycardic response in all insulin-treated groups

**Discussion**

The main findings of this study were that selective AT₁ receptor blockade with losartan did not affect the plasma...
epinephrine response during insulin-induced hypoglycemia in conscious rats, however, drugs that produced simultaneous withdrawal of both AT₁ and AT₂ receptor-mediated effects, i.e., enalapril or combined treatment with losartan + PD 123319, attenuated the reflex increase in plasma epinephrine release elicited during insulin-induced hypoglycemia in conscious rats.

The finding that selective AT₁ receptor blockade alone did not attenuate the epinephrine response in conscious rats is in accordance with our previous findings in healthy humans. However, the present in vivo findings disagree with two in vitro studies on isolated perfused rat adrenal glands which suggested that losartan, but not PD 123319, attenuated the epinephrine release elicited by exogenous angiotensin II. The discrepancies between these studies may be related to several differences between in vitro and in vivo studies. First of all, in the in vitro studies, the effect of angiotensin II on chromaffin cell secretion was examined, while in the present study, we investigated the effect of pharmacological blockade of the renin-angiotensin system on the sympathoadrenal reflex response, which involves both activation of glucose-sensitive areas in the central nervous system, stimulation of descending pathways from the hypothalamic region to the adrenal gland, and finally, the release of epinephrine from the adrenal medulla.

Secondly, in the in vitro studies, supraphysiological concentrations of angiotensin II were used to stimulate adrenomedullary epinephrine release and therefore the observed responses may not reflect physiological responses. Our finding that inhibition of angiotensin II formation by enalapril attenuated epinephrine release during insulin-induced hypoglycemia concurs with our findings in humans and with the findings of McIntyre et al in anesthetized rats. However, these authors were unable to reproduce their findings in conscious rats. In the present study, we demonstrated that combined AT₁ + AT₂ receptor blockade attenuated the epinephrine response during insulin-induced hypoglycemia by almost 40%. Moreover, the overall epis-
noradrenaline responses during insulin-induced hypoglycemia were similar in rats pretreated with enalapril and PD 123319. Losartan alone did not affect epinephrine release during hypoglycemia, but considering the effect of AT₂ receptor blockade on epinephrine release, it is likely that the powerful activation of renin release during AT₁ receptor blockade may cause an increased AT₂ receptor-mediated facilitation of epinephrine release, which could mask a possible effect of losartan alone. Together, these findings suggest that blockade with either enalapril or combined treatment with losartan + PD 123319 blunts the epinephrine release during insulin-induced hypoglycemia significantly. These results are compatible with a synergistic action of selective AT₁ and AT₂ receptor blockade on the sympathoadrenal reflex response during insulin-induced hypoglycemia.

The present study does not allow us to identify the anatomical site and/or cellular mechanism of the interaction between AT₁ and AT₂ receptor blockade, however, both the adrenal medulla and the central nervous system are putative sites of action of both AT₁ and AT₂ receptor antagonists AT₂ receptors constitute the major part of angiotensin II receptors in the adrenal medulla from both rat and humans. Numerous investigators have identified both AT₁ and AT₂ receptor binding sites in brain areas involved in the central regulation of cardiovascular function in several species including the rat. The majority of functional studies suggest that the pressor response to intracerebroventricular administration of angiotensin II is mediated by the AT₁ receptor alone. Furthermore, the central effects of angiotensin II on the baroreflex control of renal sympathetic nerve activity and HR appear to be mediated by AT₁ receptors. However, in young spontaneously hypertensive rats, Toney and Porter reported that combined intracerebroventricular administration of PD123319 and losartan inhibited the blood pressure response to intracerebroventricular angiotensin II more effectively than losartan alone. Moreover, Park and Henry recently demonstrated that the pressor response to intrathecal administration of angiotensin II can be blocked by intrathecal administration of either losartan or PD 123319, suggesting a role of both receptors for the pressor response to angiotensin II at the spinal level.

The differential increase in plasma epinephrine concentration (50-fold) relative to the 3-fold increase in plasma norepinephrine concentration is in agreement with insulin-induced hypoglycemia being a powerful stimulus for sympathoadrenal activation in particular. Thus, the synergistic action of AT₁, and AT₂ receptor blockade on adrenal epinephrine release may pertain specifically to situations with activation of neural pathways involved in the hypothalamic-adrenal reflex response during hypoglycemia.

In summary, this study confirmed our previous findings in humans that the epinephrine response elicited during insulin-induced hypoglycemia is blunted by CRI, but unaffected by selective AT₁ receptor blockade with losartan. This suggests that angiotensin II plays a similar role for the adrenomedullary reflex response during hypoglycemia in both rats and humans. Furthermore, combined treatment with losartan and PD 123319 proved more effective in attenuating the reflex increase in plasma epinephrine concentration during hypoglycemia than either of the two drugs when given alone. Because angiotensin II may increase adrenomedullary reflex response by stimulation of either AT₁ or AT₂ receptors in the brain, in the spinal cord, and at the level of the adrenal medulla, we speculate that combined AT₁ and AT₂ receptor blockade is more effective than pharmacological blockade of either receptor alone because angiotensin II acts at several levels and through both receptor subtypes on neural pathways involved in the sympathoadrenal reflex response elicited during insulin-induced hypoglycemia.

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