Combined Renin and Converting Enzyme Inhibition in Rats

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The effects of combined renin inhibition and converting enzyme inhibition on mean arterial pressure and the plasma renin-angiotensin system were studied in conscious rats. In sodium-replete rats the infusion of the renin inhibitor CP71362 (100 μg/kg/min) decreased blood pressure by 13±1 mm Hg (p<0.0001), reduced plasma renin activity to undetectable levels, but did not lower plasma angiotensin II. In rats treated chronically with enalapril (30 mg/kg/day), CP71362 decreased blood pressure by an additional 5±2 mm Hg (p<0.025) and reduced plasma renin activity and angiotensin II concentrations to undetectable levels. The effects of renin inhibition were also tested under conditions where the renin-angiotensin system was stimulated. In rats on a low sodium diet, CP71362 decreased blood pressure by 15±2 mm Hg (p<0.0001), a decrease similar to that in rats on a normal diet. Plasma renin activity was decreased below detectable limits, but plasma angiotensin II concentrations were not reduced. In rats on a low sodium diet treated chronically with enalapril, CP71362 did not further decrease blood pressure although angiotensin II levels were significantly reduced. An additive effect of combined converting enzyme and renin inhibition on blood pressure lowering and inhibition of plasma angiotensin II was found in rats anesthetized with Inactin. These studies demonstrate that 1) combined renin and converting enzyme inhibition causes a greater decrease in blood pressure and lowering of plasma angiotensin II concentrations than either agent alone, 2) the blood pressure-lowering effects of either renin inhibition or converting enzyme inhibition do not necessarily correspond to decreased plasma angiotensin II concentrations, and 3) angiotensin II measured in plasma during chronic converting enzyme inhibition is generated by a renin-dependent pathway. (Hypertension 1989;13:741-748)

Converting enzyme inhibitors are effective in lowering blood pressure in a number of clinical and experimental conditions. Despite an initial hypotensive response, some patients become refractory to the antihypertensive effects of converting enzyme inhibitors. In a recent study in rats, we found that acute converting enzyme inhibition (CEI) caused nearly complete inhibition of the angiotensin I (Ang I) pressor response and lowering of plasma angiotensin II (Ang II) concentrations, but with chronic administration there was increased pressor activity of Ang I and return of plasma Ang II levels. The mechanism for the appearance of Ang II during chronic CEI is not known. CEI is associated with reactive increases in plasma renin activity (PRA) and Ang I concentrations due to the loss of Ang II inhibition of renin release. The reappearance of Ang II in plasma with CEI could be due to the induction of alternate pathways for Ang II formation or by mass action via the classic cascade. Therefore, we hypothesized that combining a converting enzyme inhibitor with a renin inhibitor might result in additional blood pressure lowering and suppression of the plasma renin-angiotensin system. We used a rodent-active renin inhibitor and enalapril to study the combined effects of renin inhibition and CEI on blood pressure and the plasma components of the renin-angiotensin system in rats. Since CEI stimulates the concentration of early components of the renin cascade and Ang II antisera cross-react to variable degrees with Ang I, it was necessary to use a high-performance liquid chromatography (HPLC)-controlled radioimmunoassay to accurately measure Ang I and Ang II. The aims of the present study were 1) to study the effects of renin inhibition on mean arterial pressure (MAP)
and the renin-angiotensin system in rats, 2) to
determine the effects of combined renin inhibition
and CEI on blood pressure and the renin-
angiotensin system, and 3) to study the mecha-
nism for Ang II generation during chronic CEI.

Materials and Methods

All chemicals were of the purest commercial
grade available. Ang I, [Ile²]Ang II, and angioten-
sin III (Ang III) were purchased from Sigma
Chemical Company (St. Louis, Missouri). Chromo-
matographically pure Ang II-(3-8) hexapeptide,
Ang II-(4-8) pentapeptide, and Ang II-(5-8) tetra-
peptide were generously supplied by Dr. Mahesh
Khosla of the Cleveland Clinic (Cleveland, Ohio).
[¹²⁵I]Ang I and [¹²⁵I]Ang II were purchased from E.I.
DuPont de Nemours & Co., Inc./NEN Prod-
ucts (Boston, Massachusetts). CP71362 [Boc-
Phe-His-(2R,4S,5S)-5-cyclohexylmethyl-
4-hydroxy-2-isobuty1-5-aminopentany1-Lys-
Phe diacetate] was synthesized by Pfizer, Inc.
(Groton, Connecticut). Enalapril maleate was pro-
vided by Dr. Charles Sweet of the Merck Institute
for Research (West Point, Pennsylvania).

Experiments were performed on 117 adult male
Sprague-Dawley rats (Charles River Laboratories,
Wilmington, Massachusetts), weighing 200-250 g at
the start of the study protocols. All protocols were
in accordance with institutional guidelines for the
care and handling of laboratory animals. The rats on
a normal diet were given ad libitum access to
a normal diet were given ad libitum access to
standard Purina rat chow (0.42% sodium ash con-
tent) and tap water. Rats on a low sodium diet were
given a commercially prepared diet (no. 902902,
standard Purina rat chow (0.42% sodium ash con-
tent) and tap water. Rats on a low sodium diet were
given a commercially prepared diet (no. 902902,
Ainsworth, Illinois) and tap water. Rats on a low sodium diet were
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Ainsworth, Illinois) and tap water.

Blood Collection

Rats were killed by rapid decapitation, and blood
was collected for angiotensins and PRA into heparin-
coated beakers containing ethylenediaminetetraace-
tic acid (14 mg/100 μl). About 0.5 ml blood was
collected separately to obtain serum for ACE activity,
which was measured with a commercial kit
(Ventrex Labs., Portland, Maine). After centrifugation, 0.5 ml plasma was frozen and assayed for PRA
by the method of Sealey and Laragh. Thirty micro-
liters of 5% disopropyl fluorophosphate in isopropyl
alcohol was added to the remaining plasma.

Extraction of Plasma

Plasma was extracted with phenylisilyl-silica col-
umns (Bond Elut, Analytech International, Har-
bor City, California). Approximately 1,000 cpm of
[¹²⁵I]Ang II was added to each sample as a recovery
marker. Columns were conditioned with methanol
(1 ml) and water (1 ml), samples were applied (2 ml),
and the loaded columns were washed with water (1
ml) and eluted with methanol (0.5 ml). The extract
was filtered (0.45 μm filters, Acrodisc, Gelman,
Ann Arbor, Michigan) and dried.

High-Performance Liquid Chromatography

Separation of Angiotensins

The HPLC separation of angiotensins was per-
formed as previously reported from our laboratory4
incorporating the modifications described by Husain
et al. The extracted residue was dissolved in 60 μl
mobile phase for HPLC. The separation of angioten-
sins was achieved using a 3.9 mm×15 cm Nova-Pak
C18 column (Waters Chromatography Div., Mil-
ford, Massachusetts) connected to a Perkin-Elmer
(Norwalk, Connecticut) Series 4 pump system with
a Rheodyne 7125 manual injection valve (Rheodyne
Inc., Cotali, California). Elution was achieved using
a nonlinear gradient (Gradient 2) from 89% buffer A
(25 mM sodium phosphate and 5% acetonitrile, pH
7.8) and 11% buffer B (95% acetonitrile) to 68% buffer A over 12 minutes at a flow rate of 1.5 ml/min.
Fractions (6 seconds) were collected into 12×75 mm
glass test tubes and dried. The positions of angioten-
sin peaks were determined by injection of approxi-
mately 1 μg of Ang I, Ang II, Ang III, angiotensin
(3-8) hexapeptide and angiotensin(4-8) pentapep-
tide and monitored by absorption at 210 nm. Retention
times for Ang I, Ang II, Ang III, angiotensin(3-8)
hexapeptide and angiotensin(4-8) pentapeptide,
and [¹²⁵I]Ang II were 12.9, 8.3, 10.3, 11.5, 9.1, and 11.1
minutes, respectively. The appropriate fractions
were collected for the determination of Ang I and
Ang II by radioimmunoassay.
Radioimmunoassay

Ang I and Ang II were measured as previously described for our laboratory using separate radioimmunoassays for Ang I and Ang II. The characteristics of these assays have been previously published. In brief, the appropriate fractions were reconstituted with 0.5 ml assay diluent and antibody added. The Ang I assay was incubated for 24 hours before addition of [125I]Ang I (approximately 1 pg), and the incubation was continued for an additional 24 hours at 4°C. The Ang II assay was incubated at 37°C for 6 hours before addition of [125I]Ang II (approximately 1 pg), and the incubation was continued for an additional 18 hours at 4°C. Bound and free hormone were separated using dextran-coated charcoal in Tris buffer, and free hormone was counted in a Packard autogamma spectrometer (Packard Instr. Co., Inc., Downers Grove, Illinois).

Protocols

The doses of enalapril and CP71362 were selected on the basis of preliminary studies. We previously demonstrated a maximal hypotensive effect of a single oral dose of enalapril (10 mg/kg) in conscious sodium-replete normal rats. MAP was not further lowered at 30 mg/kg. In preliminary studies enalapril (30 mg/kg/day) lowered MAP in rats on a sodium-restricted diet for 1 week (sodium-restricted diet, 103±3 mm Hg, n=11; sodium-restricted diet+enalapril, 62±4 mm Hg, n=6; p<0.01). The acute administration of additional enalapril (100 mg/kg i.v.) did not further lower MAP (62±3 mm Hg). CP71362 (100 µg/kg/min) decreased blood pressure in normal conscious rats by 13±1 mm Hg (n=11). At this dose, PRA was undetectable. Due to the limited availability of CP71362, we did not attempt to determine the maximal hypotensive dose of CP71362.

In the first protocol the effects of acute renin inhibition with CP71362 on blood pressure and the plasma components of the renin-angiotensin system were studied in conscious, chronically catheterized rats maintained on a normal sodium diet. After stabilization of blood pressure, the renin inhibitor CP71362 was infused at a rate of 100 µg/kg/min for 15 minutes. CP71362 was dissolved in a vehicle consisting of 94.8% Intralipid (10% i.v. fat emulsion, KabiVitrum, Inc., Alameda, California), 5% EtOH and 0.2% 1N HCl. At the end of the infusion, rats were immediately decapitated, and blood was collected for measurement of the plasma renin-angiotensin system (renin inhibition alone). A separate group of rats was infused for 15 minutes with vehicle only as a control (vehicle control).

The effects of adding acute renin inhibition to chronic CEI were studied in rats on a normal sodium diet. Enalapril (300 mg/l) was administered in the drinking water for 1 week before measurements were taken. Chronic catherers were placed on day 5. On day 7 CP71362 (CEI+renin inhibition) or vehicle (CEI alone) was infused, and measurements were made as described above.

To determine whether the effects of renin inhibition were dependent on the duration of CEI, the effects of CP71362 were studied in additional rats 3 hours after a single dose of enalapril (30 mg/kg p.o.) (protocol 2).

In a third protocol the effects of general anesthesia (Inactin, 100 mg/kg i.p., Andrew Lockwood Associates, East Lansing, Michigan) on the blood pressure response to CEI alone or in combination with renin inhibition were studied. Rats were maintained on a normal sodium diet and dosed with enalapril for 1 week. CP71362 or vehicle was infused at 100 µg/kg/min for 15 minutes as in the first protocol.

In the fourth protocol the effects of sodium restriction on the blood pressure response to renin inhibition were studied in rats with and without chronic CEI. All of the rats were placed on a low sodium diet for 1 week before the start of experiments. Half of the rats received enalapril (300 mg/l) in their drinking water. After the placement of chronic catheters, CP71362 or vehicle was infused as described in the first protocol.

Vehicle infusion had no effect on any of the variables measured in any of the protocols.

Statistics

The data are expressed as the mean±SEM as the index of dispersion. Data were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls method for multiple comparisons. Student's t test for paired or unpaired data was used where appropriate. Differences were considered significant when p<0.05.

Results

Normal Diet, Conscious

In normal, conscious rats the renin inhibitor CP71362 (100 µg/kg/min for 15 min) decreased blood pressure by 13±1 mm Hg (MAP 101±3 to 88±3 mm Hg, n=11, p<0.0001) (Figure 1). Measurements of the plasma components of the renin-angiotensin system are shown in Figure 2. After infusion of the renin inhibitor, PRA was below the detection limit of the assay (normal, 4.1±0.4, n=7; CP71362, <0.2 ng Ang I/ml/hr, n=6, p<0.01). The renin inhibitor did not change ACE activity (normal, 186±23, n=4; CP71362, 138±21 nmol/ml/min, n=6). Plasma Ang II levels were not affected by the renin inhibitor (normals, 24.4±8.4, n=6; CP71362, 37.5±12.5, n=6).

When enalapril was administered for 1 week to rats on normal sodium diet, MAP was decreased by 18 mm Hg (normal, 101±3, n=11, vs. enalapril, 83±4, n=9, p<0.01) (Figure 1). As shown in Figure 2, PRA was not changed (normal, 4.1±0.4, n=7, vs. enalapril, 4.5±0.7, n=10), ACE activity was decreased to below the detection limit (<15
The effects of combined CEI and renin inhibition on plasma renin-angiotensin system were studied in conscious, sodium-replete rats. PRA, plasma renin activity; ACE, angiotensin converting enzyme activity; ANG II, plasma angiotensin II concentration; ND, no drug; RI, renin inhibition (CP71362, 100 μg/kg/min i.v.); CEI, converting enzyme inhibition (enalapril, 30 mg/kg/day x 7 days); CEI+RI, combined converting enzyme inhibition and renin inhibition.

Normal Diet, Inactin Anesthesia

The effects of general anesthesia on the responses to combined CEI and renin inhibition were studied in additional experiments. In rats treated for 1 week with enalapril and anesthetized with Inactin, the infusion of CP71362 decreased blood pressure by 8±1 mm Hg (MAP 73±4 to 65±3 mm Hg, n=11, p<0.0001) (Figure 4). PRA was decreased below the limit of detection (enalapril alone, 4.2±1.4, n=5; enalapril+CP71362, <0.2 ng Ang I/ml/h, n=6, p<0.0025). As anticipated, plasma ACE levels were below detectability limits in all rats treated with enalapril. The combination of chronic enalapril and acute renin inhibition decreased plasma Ang II concentrations below detection limits in enalapril-treated rats (enalapril alone, 1,613±98, n=5; enalapril+CP71362, <15 pg/ml, n=6, p<0.0001). As anticipated, plasma ACE levels were below detectability limits in all rats treated with enalapril. The combination of chronic enalapril and acute renin inhibition decreased plasma Ang II levels below the detection limit of the assay (chronic enalapril, 24.2±6.9, n=5; enalapril+CP71362, <0.4 pg/ml, n=6, p<0.01).

Low Sodium Diet, Conscious

In rats fed a low sodium diet for 1 week, renin inhibition decreased blood pressure by 15±2 mm Hg.
(MAP 103±3 to 89±4, n=11, p<0.0001) (Figure 5). Measurements of the plasma components of the renin-angiotensin system are shown in Figure 6. In the presence of CP71362, PRA was decreased below the limit of detection (low sodium alone, 5.2±1.4, n=6; low sodium+CP71362, <0.2 ng Ang I/ml/hr, n=11). CP71362 decreased plasma Ang I concentrations (low sodium, 304±146, n=6; low sodium+CP71362, <15 pg/ml, n=10, p<0.05). Renin inhibition did not affect plasma ACE activity (low sodium, 174±39, n=4; low sodium+CP71362, 138±26 nmol/ml/min, n=10, p=NS) but lowered plasma Ang II concentrations by 78% (low sodium alone, 129.7±75.3, n=6; low sodium+CP71362, 28.0±7.2 pg/ml, n=9, p<0.05). The effects of adding a renin inhibitor to rats fed low sodium diet and given enalapril for 1 week were studied. The addition of the renin inhibitor to rats treated with a converting enzyme inhibitor did not further reduce blood pressure (MAP, 68±4 to 64±3 mm Hg, n=11, p=NS) (Figure 5), although PRA was reduced (low sodium, enalapril, 6.2±1.4, n=10; low sodium, enalapril+CP71362, <0.2 ng Ang I/ml/hr) (Figure 6). CP71362 decreased plasma Ang I concentrations by >99% (low sodium, enalapril, 2,423±604, n=8; low sodium, enalapril+CP71362, 22.8±7.1 pg/ml, n=10, p<0.01). Plasma ACE activity was not detectable in either group. In the rats given CEI alone, there was a significant increase in plasma Ang II levels (low sodium, 130±75, n=6; low sodium, enalapril, 335±93 pg/ml, n=8, p<0.05). Plasma Ang II concentrations were decreased to very low levels after renin inhibition (low sodium, enalapril, 335±93, n=8; low sodium, enalapril+CP71362, 4.3±1.2 pg/ml, n=10, p<0.01).

Discussion

Pharmacological inhibition of the renin-angiotensin system with angiotensin converting enzyme inhibitors has proven to be an effective means of controlling hypertension in many, but not all, patients. In a previous study we reported a sustained hypotensive effect of enalapril in rats dosed for up to 2 months. However, plasma Ang II levels returned toward normal, and the pressor response to infused Ang I increased progressively with time. The mechanism for formation of Ang II was not clear. Ang II could be generated from Ang I by incompletely inhibited converting enzyme, by other enzymes such as tonin, or directly from angiotensinogen by sequential cleavages. Alternatively, ACE inhibitors may not inhibit all pools of ACE, and the intracellular renin-angiotensin system may not be inhibited. Recently, there has been substantial interest in the development of renin inhibitors. The present investigation was performed to test the hypothesis that inhibition of the renin-
angiotensin system at two sites would result in further reductions in blood pressure and suppression of plasma angiotensins.

The acute infusion of the renin inhibitor CP71362 decreased blood pressure an equal amount in conscious, sodium-replete and sodium-restricted rats. In both of these groups PRA was reduced below the detection limit of the assay, and plasma Ang I concentrations were decreased by at least 90%. Plasma Ang II was not lowered in sodium-replete animals but was lowered from elevated levels (due to sodium restriction) into the normal range in the rats on sodium-restricted diets. In rats acutely treated with enalapril at a dose sufficient to cause a maximal blood pressure lowering and a suppressed plasma Ang II,1 CP71362 caused a further decrease in blood pressure. These results demonstrate that the blood pressure-lowering effects of acute renin inhibition with CP71362 do not depend on measurable levels of plasma Ang II.

The combined administration of an angiotensin converting enzyme inhibitor and renin inhibitor further lowered blood pressure in rats on a normal sodium diet. Rats maintained on a sodium-restricted diet and chronically dosed with enalapril were hypotensive, and blood pressure was not further reduced by acute renin inhibition. The combined treatment with a converting enzyme inhibitor and a renin inhibitor decreased PRA and plasma Ang II concentrations below the limit of detection, although either inhibitor alone (chronic enalapril or acute CP71362) did not decrease plasma Ang II concentrations. The effects of combined renin inhibition and CEI to substantially reduce PRA, plasma ACE, Ang I, and Ang II levels were consistent and occurred in acute and chronic experiments, in sodium-replete and sodium-restricted states, and in the presence of general anesthesia. These data suggest that inhibiting the renin-angiotensin system at two sites (ACE and renin) can be more effective in lowering blood pressure and suppressing the plasma renin-angiotensin system than either inhibitor alone.

Several investigators have examined the effects of renin inhibition on blood pressure using a variety of animal models.14–25 Renin inhibitors have generally been effective in reducing PRA and lowering blood pressure during states of sodium depletion.14,16,20,22,23 Renin inhibitors have also been reported to increase immunoreactive plasma renin.25 Renin and converting enzyme inhibitors caused similar reductions in blood pressure after acute or chronic administration in salt-depleted normotensive marmosets17,23–25 and renal hypertensive dogs.13 In acute experiments Tree et al19...
measured the effects of renin inhibition on plasma Ang II and found that there was a temporal correlation between the fall in blood pressure and reduction in plasma immunoreactive Ang II. However, this temporal association may not be indicative of mechanism. In a recent study we examined the effects of chronic renin inhibition on the plasma renin-angiotensin system in cynomolgus monkeys. Plasma Ang II levels were reduced initially but returned toward control despite continued blood pressure lowering and inhibition of PRA. Therefore, although there may be a good correlation between blood pressure lowering and suppression of plasma Ang II levels in acute experiments, the correlation does not necessarily occur chronically.

The effects of combined renin and ACE inhibition on blood pressure and the plasma renin-angiotensin system were addressed previously. Oldham et al reported additional blood pressure lowering by CEI in sodium-deficient dogs treated with a renin inhibitor. Blaine et al compared the effects of acute renin inhibition and CEI in sodium-deficient dogs and found that circulating Ang II levels were reduced in a dose-related manner, although blood pressure was maximally inhibited at a time when there were measurable levels of circulating Ang II. The addition of the renin inhibitor SCRIP reduced plasma Ang II to virtually zero, but blood pressure was unaffected. These data are in agreement with the present investigation when sodium-restricted animals were studied. Although the exact explanation for the dissociation between blood pressure reduction and plasma Ang II concentrations is not clear, it is possible that plasma Ang II levels do not necessarily reflect tissue activity of the renin-angiotensin system, which may more closely correlate with blood pressure.

Several interesting findings were noted in the sodium-restricted rats. The chronic administration of enalapril did not result in significant elevations in PRA but did cause plasma Ang II levels to rise. In a previous study we reported that PRA acutely increased in rats treated with enalapril but returned toward control by 1 week. Chronic CEI was associated with normal to elevated plasma Ang II levels. It is possible that the reactive rise in PRA was inhibited by the reappearance of Ang II in plasma (short feedback loop). The source of the plasma Ang II during chronic CEI has also been previously examined. Although standard assays for ACE indicate virtually complete suppression of the enzyme in plasma in rats chronically treated with enalapril, the pressor response to Ang I is restored by 60% compared with virtually complete inhibition acutely. These data are compatible with the generation of plasma Ang II from Ang I during chronic CEI either by classical pathways (incomplete inhibition of ACE at tissue sites) or by alternate pathways.

In conscious rats on normal sodium diet, Ang II levels were not lowered after acute renin inhibition despite reductions in blood pressure and PRA. Ang II formation clearly occurred despite undetectable PRA. There are several possible explanations for this finding: 1) Ang I formation may occur by non-renin-dependent pathways. 2) Plasma levels of PRA and Ang II may not adequately reflect the activity of the tissue renin-angiotensin system (compartmentalization). 3) PRA measured in vitro may not reflect Ang I formation in vivo in the presence of transitional state analogues (renin inhibitor). The available data do not allow us to differentiate among these possibilities.

The combination of acute renin inhibition superimposed on 1 week of CEI resulted in a profound fall in Ang II levels despite the fact that neither agent alone had any effect on Ang II levels. As discussed above, acute CEI is associated with a reactive rise in the early components of the renin-angiotensin system. With increasing duration of converting enzyme inhibitor administration to rats, the conversion of Ang I to Ang II increases, and plasma Ang II rises. Under these conditions, the addition of a renin inhibitor blocks the formation of Ang I, and there is a profound reduction in plasma Ang II levels.

The current investigation also provides insights into the mechanism of Ang II formation during chronic CEI. If Ang II is formed from angiotensinogen without involvement of ACE, the superimposition of renin inhibition onto CEI should not lower plasma Ang II. If Ang II is formed by mass action from increased Ang I levels (which occur during CEI) or by alternate enzymes, which cleave Ang I to Ang II, the superimposition of a renin inhibitor may block Ang II formation by lowering Ang I concentrations. These experiments clearly demonstrate substantial reductions in plasma Ang II during combined inhibition (often to below detectability levels), which argues strongly against the former pathway. These experiments cannot distinguish between a mass action effect via residual converting enzyme activity or the role of alternate enzymatic pathways for Ang I conversion during chronic CEI. We did not have sufficient amounts of renin inhibitor to test the effects of higher doses on Ang II formation or to test the effects of adding acute CEI to chronic renin inhibition.

In summary, blood pressure and the components of the plasma renin-angiotensin system were measured in rats treated with CEI, renin inhibition, or both. When a renin inhibitor was given to rats acutely or chronically treated with converting enzyme inhibitor, there was an additional hypotensive effect in the sodium-replete but not in sodium-restricted state. In all groups of animals receiving the renin inhibitor alone or in combination with CEI, PRA was suppressed. Only combined administration of CEI and renin inhibition reduced plasma Ang II levels to virtually zero. Although the hypotensive effects of renin and converting enzyme inhibitors did not always correlate with plasma Ang II.
levels, the demonstration that combined renin and ACE inhibition further lowers blood pressure in sodium-replete rats supports the notion that these agents, when used together, may further lower blood pressure when either agent alone is not effective in controlling hypertension.

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References


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