Clearance of Human Brain Natriuretic Peptide in Rabbits; Effect of the Kidney, the Natriuretic Peptide Clearance Receptor, and Peptidase Activity

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ABSTRACT
Although the synthetic version of the cardiac peptide human brain natriuretic peptide (hBNP) has demonstrated beneficial cardiovascular effects in clinical studies, little is known about mechanisms governing its elimination from the blood. This study measured the role of the kidney, the natriuretic peptide clearance (NP-C) receptor, and peptidase digestion on the elimination of synthetic hBNP from the plasma compartment of rabbits. The estimated plasma steady state resulting from a continuous i.v. infusion was achieved within 50 min and was related in a linear manner with the infusion rate of the drug. Complete restriction of kidney blood flow by bilateral suture-ligation of the renal arteries compared with sham-treated animals reduced the clearance of hBNP by approximately half (24 ± 9 ml/min versus 47 ± 14 ml/min, respectively, p < .007). Pharmacological blockade of the NP-C receptor with a clearance receptor-specific analog of atrial natriuretic peptide increased in a statistically significant and dose-related manner the plasma steady-state level of hBNP during continuous i.v. infusion of hBNP (maximum effect of 1.9 ± 0.3-fold, p < .01). The peptidase inhibitor phosphoramidon increased in a dose-related manner the plasma steady-state level of hBNP 1.7 ± 0.4-fold during continuous i.v. infusion of hBNP in rabbits. These data suggest that the kidney, the NP-C receptor, and peptidases are all important in the elimination of hBNP from the plasma compartment.

Human brain natriuretic peptide (hBNP) is a cardiac derived peptide hormone that regulates cardiovascular and renal function (Lewicki, 1995). A synthetic version of endogenous hBNP with an identical amino acid sequence, termed Natrecor, has beneficial effects in patients with congestive heart failure reflected by reduced pulmonary capillary wedge pressure, reduced right atrial pressure and increased cardiac output (Hobbs, 1996; Marcus, 1996; Mills, 1997; Abraham, 1998). The purpose of the studies described here was to assess pathways involved in the elimination of exogenous hBNP from the plasma compartment. As renal blood flow may be impaired in some congestive heart failure patients who may receive Natrecor, the role of the kidney in the elimination of plasma hBNP may be particularly important and was included in this study. There are at least two biochemical mechanisms that might mediate the elimination of hBNP from the plasma compartment: the natriuretic peptide clearance (NP-C) receptor and peptidase digestion. The NP-C receptor mediates the cellular internalization and subsequent lysosomal degradation of the peptide hormone atrial natriuretic peptide (ANP; Nussenzveig, 1990) and presumably plays a similar role for the structurally related peptide hBNP. It is known that hBNP binds with high affinity to the NP-C receptor (Bennett, 1991). It has been shown that truncated analogs of rat ANP bind with significantly higher affinity to the NP-C receptor than to the guanylyl cyclase-A receptor (Maack, 1987), a known biological receptor for both hBNP and ANP. One NP-C receptor-specific peptide, rat ANP(4–23), has been used to assess the role of the NP-C receptor in the metabolism of ANP in animals (Almeida, 1989). In this report we describe studies that use an NP-C receptor specific analog, des(18–22)human ANP-(4–23)-NH₂ (Scarborough, 1986) to assess the role of the NP-C receptor in the metabolism of hBNP in rabbits. It is known that rabbit tissue expresses the NP-C receptor (Murchy, 1998). Previous studies in sheep with this NP-C receptor ligand have shown that it increases plasma levels of endogenous ANP and BNP (Charles, 1996; Rademaker, 1997). It is known that hBNP is a substrate for neutral endopeptidase (NEP) 24.11 purified from rat (Norman, 1991; Kenny, 1993). In this report we assess the role of peptidases in the elimination of plasma hBNP in rabbits using the peptidase
inhibitor phosphoramidon. Phosphoramidon has been shown to be an inhibitor of NEP24.11 (Roques, 1990).

The role of the kidney in the elimination of plasma hBNP was investigated as this organ might clear the peptide by filtration or by metabolism. It is known that the NP-C receptor (Maack, 1987) and NEP24.11 (Sonnenberg, 1988) are abundant in the kidney and might be involved in metabolism of hBNP in this organ. The effect of complete renal blood flow restriction induced by bilateral renal artery suture ligation on the pharmacokinetics of hBNP in rabbits was assessed.

### Experimental Procedures

#### Materials

The NP-C receptor-specific peptide used in these studies is an analog of human ANP in which part of the ring structure as well as the carboxy-terminal tail portion is deleted: Arg-Ser-Ser-Cys-Gly-Ang-Met-Asp-Arg-Ile-Gly-Ala-Cys-amide (Scarborough, 1986), termed C-ANP, and was prepared at Scios Inc. using standard peptide synthesis methodologies. Aprotinin and phosphoramidon/N-((rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp, were purchased from Sigma Chemical Co. (St. Louis, MO) and lidocaine HCl, (Xylocaine Jelly) was purchased from Astra (Westborough, MA).

#### Methods

**Male New Zealand White rabbits (R & R Rabbitry, Standwood, WA) weighing between 2.5 and 3.0 kg were used in the study. The animals were allowed to acclimate at the animal facility for at least 1 week before use.**

During the study, the rabbits were weighed and placed in Plexiglas restrainers, and both ears were shaved and swabbed with 70% isopropyl alcohol and then 2% lidocaine HCl before catheter placement. Venous catheters (Becton Dickinson, Sunny, UT) fitted with a heparin lock were inserted into the intermedial branch of the peripheral ear vein of both ears for drug administration and initiation and maintenance of anesthesia, respectively. A catheter was also placed in the central ear artery for blood sampling.

#### Pharmacokinetics of hBNP in Rabbits

**Conscious rabbits (n = 6) received an i.v. infusion of vehicle for a 60-min control period followed by an escalating dose of hBNP (0.05, 0.1, and 0.2 μg/kg/min) for 60 min at each dose. Blood samples (2 ml) were taken during the control period (time 0) and 50, 55, and 60 min after the initiation of each dose of hBNP.**

Thus, blood samples were taken at 0 min (vehicle), 50, 55, and 60 min (0.05 μg/kg/min hBNP), 110, 115, 120 min (0.1 μg/kg/min hBNP), and 170, 175, and 180 min (0.2 μg/kg/min hBNP). Blood was collected into EDTA tubes containing 150 kallikrein-inactivating units aprotinin, centrifuged immediately, and plasma samples were stored frozen at −80°C until assayed for hBNP. Plasma hBNP was determined as described below. An estimated plasma steady-state value associated with each dose was the mean of the values derived from blood drawn at the three time points.

#### Complete Restriction of Renal Blood Flow

For studies involving bilateral renal artery ligation, the animals were anesthetized with 40 to 60 mg/kg pentobarbital. Once under anesthesia, the animals were removed from the restrainers, the abdominal area was shaved and the animal was positioned on its side on a table lined with a heating pad. The temperature was maintained at 37°C for the duration of the experiment. A surgical plane of anesthesia was attained by a constant infusion of 10 mg/kg/h of pentobarbital. Access to the kidneys was through an incision made on the dorsal lateral side of the abdomen. The abdominal muscle was bluntly dissected to gain entry into the peritoneal cavity and expose the kidney. The renal artery was dissected free of fatty tissue and ligated proximally and distally with 3 to 0 suture. The kidney was positioned back into the peritoneal cavity and the wound closed with stainless steel staples. The same procedure was performed on the contralateral kidney. Control animals underwent the same surgical manipulations with the exception of actual renal artery ligation (sham procedure). hBNP was administered as a continuous infusion at a dose of 0.1 μg/kg/min, which was initiated 60 min before the renal artery ligation and continued for another 60 min after the surgical procedure was completed. Blood samples were drawn at −20, 0, 50, 55, 60, 110, 115, and 120 min into the infusion to measure steady-state plasma levels of hBNP before and after ligation of the renal arteries. Blood volume was replaced at each time point with an equal volume of 0.9% NaCl. Plasma was collected as described above and immunoreactive-hBNP determined as described below. An estimated plasma hBNP steady-state value for each animal before and after the surgical procedure (ligation or sham) was derived from blood drawn at the three time points. There were six animals per experimental group.

In a second study, hBNP was administered as an i.v. bolus dose of 10 μg/kg to animals with bilateral suture ligation of the renal arteries and to sham-operated animals. Blood samples (3 ml) were drawn at the following time points: −20, 0, 5, 15, 30, 60, 90, and 120 min posttreatment. All surgical procedures preceded hBNP administration. Plasma was collected as described above and immunoreactive-hBNP was determined as described below. Plasma hBNP values were fitted to a two-compartment model assuming drug concentrations decline biexponentially as the sum of two first-order processes as described by the formula: 

\[ C_t = A \exp(-\alpha \cdot t) + B \exp(-\beta \cdot t) \]

Values for \( T_{1/2a} \) and \( T_{1/2b} \) were calculated from 0.693/α and 0.693/β, respectively and values for clearance (CL) were determined from the formula: 

\[ \text{CL} = \frac{\text{hBNP dose} \times \text{AUC}_{0-\infty}}{\text{steady-state}} \]

with \( \text{AUC}_{0-\infty} \) with \( \text{AUC}_{0-\infty} = \text{AUC} \times e^{-\beta t} \text{h} \times \text{hBNP}^{2/3} \) where \( \text{hBNP}^{2/3} \) is the measured plasma concentration of hBNP at 2 h (Ritschel, 1992).

#### NP-C Receptor and Peptidase Inhibition

The roles of NP-C receptor and peptidases in the elimination of plasma hBNP were evaluated in conscious rabbits. hBNP was administered to rabbits as a continuous infusion at a dose of 0.02 μg/kg/min for a total of 4 h. One hour after the initiation of the hBNP infusion, C-ANP was infused at escalating doses of 0.1, 1.0, and 10.0 μg/kg/min for 1 h at each dose. Blood samples were drawn at −20, 0, 80, 85, 90, 140, 145, 150, 200, 205, 210, 260, 265, and 270 min into the hBNP plus C-ANP infusion. In studies involving the use of the neutral endopeptidase inhibitor, phosphoramidon, hBNP was infused at a dose of 0.02 μg/kg/min for a total of 4 h. Phosphoramidon infusion at a dose of 25 μg/kg/min was begun 1 h after the initiation of the hBNP infusion. After an additional hour, the phosphoramidon dose was increased to 50 μg/kg/min and treatments continued for 2 h. C-ANP, 10 μg/kg/min, was added at the last hour of infusion. During this final hour, the rabbits were being infused simultaneously with hBNP, phosphoramidon, and C-ANP. Blood samples were drawn at the following time points to reflect each drug’s steady-state level (using the start of hBNP infusion as time 0): 40, 50, 60, 100, 110, 120, 160, 170, 180, 220, 230, and 240 min. In the same study, a separate set of rabbits was used as control group receiving only hBNP and C-ANP at the last hour of the 4-h infusion.

#### Plasma hBNP Assay

Plasma immunoreactive hBNP was determined by immunossay as described previously (Clemens, 1998). Briefly, plasma samples were diluted appropriately with pooled normal rabbit plasma. hBNP-specific monoclonal antibodies were added to the samples and incubated overnight in microtiter wells precoated withFc-specific antimurine monoclonal antibodies. The amount of hBNP bound relative to the total binding capacity of the monoclonal antibody was determined by measuring the binding of biotinylated hBNP. Biotinylated hBNP was quantitated using avidin-horseradish peroxidase with a tetramethyl benzidine substrate, which allows for a colorimetric endpoint.

#### Statistical Analysis

Plasma hBNP concentrations and steady-state values are expressed as the mean ± S.D. The significance of complete renal blood flow restriction or the sham procedure on steady-state plasma hBNP was assessed by comparing steady-state levels achieved by continuous hBNP infusion before and after these procedures using a paired Student’s t test with P < .05 considered statistically significant. The effect of complete renal blood flow restriction on the fold increase in steady-state hBNP values relative to sham was assessed using an unpaired Student’s t test with P < .05 considered statistically significant.

The significance of differences in the pharmacokinetics of hBNP

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when administered by i.v. bolus to animals with complete renal blood flow restriction and sham procedure was assessed using an unpaired Student’s t test with \( P < .05 \) considered statistically significant.

The significance of the NP-C receptor blockade with various doses of C-ANP on the steady-state plasma hBNP values was assessed using repeated measures ANOVA with the Bonferroni multiple comparisons test with \( P < .05 \) considered statistically significant.

The significance of peptidase inhibition with phosphoramidon on the steady-state plasma hBNP values was assessed using repeated measures ANOVA with the Bonferroni multiple comparisons test with \( P < .05 \) considered statistically significant.

**Results**

**Pharmacokinetics of hBNP in Rabbits.** Plasma levels of hBNP resulting from a dose-escalating protocol involving continuous i.v. infusion of 0.05, 0.1, and 0.2 \( \mu \)g/kg/min were determined at the times indicated (see Table 1). There was no significant difference between the plasma hBNP levels taken 50, 55, or 60 min after initiation of each infusion dose, suggesting a steady-state level was achieved within 50 min. There was a linear relationship \( (R^2 = 0.9999) \) between the estimated steady-state values of hBNP and the infusion dose of hBNP (see Fig. 1), with steady-state values of 2.5 \( \pm \) 0.6, 5.7 \( \pm \) 0.8, and 11.9 \( \pm \) 1.7 ng/ml for hBNP continuous infusion doses of 0.05, 0.1, and 0.2 \( \mu \)g/kg/min, respectively.

**The Effect of Kidney on the Elimination of Plasma hBNP.** The pharmacokinetics of hBNP derived from continuous i.v. infusion \((0.1 \mu \text{g/kg/min})\) was evaluated before and after complete restriction of kidney blood flow by bilateral renal artery suture ligation. The plasma hBNP levels are shown in Table 2. The steady-state plasma hBNP values increased in a statistically significant manner \((p < .01)\) from 5.2 \( \pm \) 2.0 ng/ml before the surgical procedure to 9.7 \( \pm \) 3.9 ng/ml after suture ligation \((1.9 \pm 0.4\text{-fold})\). In contrast, there was no significant change in the steady-state plasma hBNP values before and after the sham procedure, 6.0 \( \pm \) 1.8 ng/ml and 6.2 \( \pm \) 2.5 ng/ml, respectively \((1.1 \pm 0.4\text{-fold})\).

In the second protocol, plasma hBNP levels were determined after a 10 \( \mu \)g/kg i.v. bolus to animals with renal artery ligation and animals with a sham kidney procedure (see Fig. 2). Plasma hBNP levels were statistically higher in animals subjected to renal artery occlusion compared with the values obtained from sham-operated animals. There was a statistically significant difference in the clearance values for hBNP obtained from animals with complete blood flow restriction and sham-treated animals, 24 \( \pm \) 9 and 47 \( \pm \) 14 ml/min, respectively.

**TABLE 1**

<table>
<thead>
<tr>
<th>Plasma hBNP concentration (ng/ml)</th>
<th>0.05 ( \mu )g/kg/min</th>
<th>0.1 ( \mu )g/kg/min</th>
<th>0.2 ( \mu )g/kg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>2.7 ( \pm ) 0.5</td>
<td>5.9 ( \pm ) 1.0</td>
<td>11.3 ( \pm ) 1.5</td>
</tr>
<tr>
<td>50</td>
<td>2.4 ( \pm ) 0.2</td>
<td>5.8 ( \pm ) 1.0</td>
<td>12.4 ( \pm ) 1.8</td>
</tr>
<tr>
<td>60</td>
<td>2.3 ( \pm ) 0.04</td>
<td>5.4 ( \pm ) 1.2</td>
<td>11.9 ( \pm ) 2.3</td>
</tr>
</tbody>
</table>

Plasma hBNP concentrations during continuous i.v. infusion of hBNP in conscious rabbits \((n = 6)\) at 0.05, 0.1, and 0.2 \( \mu \)g/kg/min initiated at 0, 60, and 120 min, respectively.

**Fig. 1.** Effect of hBNP infusion rate on the steady-state plasma level of hBNP. Conscious rabbits \((n = 6)\) received hBNP by a continuous dose-escalating protocol for 60 min per dose \((0.05, 0.1, \text{and} 0.2 \mu\text{g/kg/min})\). Steady-state plasma hBNP values \(\pm\) S.D. are indicated.

**TABLE 2**

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Bilateral Sham Ligation Plasma hBNP (\text{ng/ml})</th>
<th>Bilateral Renal Artery Ligation Plasma hBNP (\text{ng/ml})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.06 ( \pm ) 0.03</td>
<td>0.05 ( \pm ) 0.08</td>
</tr>
<tr>
<td>50</td>
<td>4.8 ( \pm ) 1.5</td>
<td>5.3 ( \pm ) 2.2</td>
</tr>
<tr>
<td>55</td>
<td>7.3 ( \pm ) 2.8</td>
<td>5.2 ( \pm ) 2.7</td>
</tr>
<tr>
<td>60</td>
<td>5.9 ( \pm ) 2.0</td>
<td>5.1 ( \pm ) 1.6</td>
</tr>
<tr>
<td>110</td>
<td>7.3 ( \pm ) 2.3</td>
<td>9.7 ( \pm ) 3.5</td>
</tr>
<tr>
<td>115</td>
<td>5.8 ( \pm ) 3.0</td>
<td>10.3 ( \pm ) 4.1</td>
</tr>
<tr>
<td>120</td>
<td>5.4 ( \pm ) 2.9</td>
<td>8.9 ( \pm ) 4.5</td>
</tr>
</tbody>
</table>

Plasma levels of hBNP before \((\text{time} 0)\) and during continuous i.v. infusion of hBNP at 0.1 \( \mu \)g/kg/min. Bilateral renal artery ligation or bilateral sham procedure \((n = 6 \text{ per group})\) was performed immediately after drawing the 60-min sample.

**Fig. 2.** Pharmacokinetics of hBNP after a 10 \( \mu \)g/kg i.v. bolus in rabbits immediately after bilateral renal artery ligation or a sham surgical procedure \((n = 4 \text{ per group})\). Plasma hBNP concentrations \(\pm\) S.D. are indicated. *\(p < .05\) comparing data obtained from the two groups at each time point.

respectively \((p < .007)\). Half-life values \((T_{1/2a} \text{ and } T_{1/2b})\) were not statistically different between animals with complete blood flow restriction and sham-treated animals \((T_{1/2a} = 6.9 \pm 1.4 \text{ min and } 5.4 \pm 1.3 \text{ min, respectively, and } T_{1/2b} = 36 \pm 10 \text{ min and } 33 \pm 5 \text{ min, respectively}).
The Effect of the ANP Clearance Receptor on the Elimination of Plasma hBNP. The effect of the NP-C receptor on the elimination of plasma hBNP was evaluated with the use of the NP-C receptor-specific peptide agonist C-ANP. In animals administered i.v. hBNP by continuous infusion (0.02 μg/kg/min), plasma hBNP levels were determined before and after coadministration of C-ANP at doses of 0.1, 1.0, and 10 μg/kg/min. As shown in Fig. 3, when compared with values obtained before C-ANP infusion, the steady-state plasma hBNP values increased in a statistically significant manner after infusion of C-ANP at doses of 1.0 μg/kg/min (1.4 ± 0.2-fold increase) and 10 μg/kg/min (1.9 ± 0.3-fold) but not at a dose of 0.1 μg/kg/min (1.0 ± 0.2-fold).

The Effect of Peptidases on the Elimination of Plasma hBNP. The effect of peptidases on the elimination of plasma hBNP was evaluated with the use of the peptidase inhibitor phosphoramidon. In animals administered i.v. hBNP by continuous infusion (0.02 μg/kg/min), plasma hBNP levels were determined before and after i.v. phosphoramidon treatment and again after combined treatment with phosphoramidon plus C-ANP. The mean steady-state plasma hBNP values increased in a statistically significant manner after 50 μg/kg/min phosphoramidon (p < .01, 1.7 ± 0.4-fold increase), but not 25 μg/kg/min phosphoramidon (see Fig. 4). The mean steady-state plasma hBNP values increased further when animals were treated with both phosphoramidon and C-ANP (p < .01, 2.4 ± 0.3-fold increase over the value derived from hBNP treatment alone; see Fig. 4). Treatment with hBNP plus C-ANP in the absence of phosphoramidon increased the mean steady-state plasma hBNP values in a statistically significant manner, 1.7 ± 0.6-fold over the values derived from hBNP treatment alone (see Fig. 4).

Discussion

This study assessed factors that influence the elimination of hBNP from the plasma compartment of rabbits including the kidney, the NP-C receptor, and peptidases. Previous studies have demonstrated that the rabbit is a useful species to study hBNP, as doses of hBNP effective in human studies yield similar hemodynamic and renal effects in rabbits (Hobbs, 1996; Clemens, 1997). The plasma concentrations of hBNP in rabbits are linearly related to the pharmacological doses used in this report. In addition, an estimated steady state was achieved within 50 min as there was consistently no significant difference in the plasma concentration of hBNP achieved 50, 55, or 60 min after initiation of infusion at all of the doses tested. The pharmacokinetics of plasma hBNP in rabbits after bolus administration is best fit by a two-compartment model with T1/2a, and T1/2b values of 5.4 ± 1.3 and 33 ± 5 min, respectively. Similar pharmacokinetic values have been previously published in a different study in rabbits (T1/2a, and T1/2b values of 5.5 ± 0.9 and 27 ± 10 min; Clemens, 1998). Beneficial effects of hBNP in human studies has been noted with doses ranging from 10 to 100 μg/kg/min (Yoshimura, 1991; Marcus, 1996; Mills, 1997; Abraham, 1998). Thus, the doses used in this animal study can be directly related to therapeutic doses.

Complete restriction of kidney blood flow reduced the clearance of hBNP by one-half, indicating that the kidney is involved in the elimination of hBNP from the blood. If this can be extrapolated to congestive heart failure patients with reduced renal blood flow, these results suggest that at worst, hBNP clearance will be reduced by half in these individuals. There is data that the kidney plays a role in the removal of BNP in humans. In a study that monitored regional plasma levels of endogenous BNP in patients with cardiac disease, a significant arteriovenous concentration gradient was found between the femoral artery and renal vein suggesting the kidney is extracting BNP in these individuals (Richards, 1993). Whether the kidney clears hBNP by filtration, proteolysis, or both, mecha-
nisms cannot be understood from these studies. It is known that the kidney is a rich source of the NP-C receptor (Maack, 1987) and NEP24.11. The role of each of these biochemical pathways in the process of renal clearance of hBNP cannot be understood from the studies presented here.

Treatment of rabbits with the NP-C receptor-specific ligand, C-ANP, significantly increased in a dose-related manner the steady-state plasma levels of hBNP resulting from a continuous i.v. infusion of hBNP. This suggests that the NP-C receptor is involved in the elimination of plasma hBNP. Previous studies utilizing the identical NP-C receptor-specific peptide used in this current study demonstrate that the NP-C receptor is involved in the clearance of endogenous ANP and BNP (Charles, 1996). However, this is the first demonstration showing that the NP-C receptor mediates the elimination of exogenous hBNP when administered at pharmacological doses. Treatment of rabbits with the peptidase inhibitor phosphoramidon significantly increased the steady-state level of hBNP. This suggests that peptidases are involved in the elimination of plasma hBNP. This hypothesis is consistent with the data demonstrating that hBNP is a substrate for NEP24.11 (Norman, 1991; Rademaker, 1997), however, whether NEP24.11 is the primary peptidase involved in this process cannot be determined from this study. The neutral endopeptidase inhibitors SCH-32615 has been shown to increase endogenous hBNP in normal sheep and with experimental heart failure (Charles, 1996; Rademaker, 1997). Furthermore, in cardiac disease patients, administration of SCH-32615 resulted in the elevation of endogenous BNP levels, suggesting that peptidases are involved in the removal of BNP in humans (Lainchbury, 1998). This report demonstrates that peptidase inhibition alters the elimination rate of exogenous hBNP administered at pharmacological doses.

In summary, these studies suggest that the kidney, the NP-C receptor, and peptidases (possibly NEP24.11) are involved in the metabolism of hBNP. However, it is not clear whether the NP-C receptor and NEP24.11 expressed in the kidney are responsible for the effects of C-ANP and phosphoramidon demonstrated in vivo as both of these proteins are found in other tissues as well as the kidney.

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References


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