Platelet Thromboxane Inhibition by Plasma Polypeptides in Prehypertensive Dahl Rats

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SUMMARY Plasma from Dahl rats susceptible to salt-induced hypertension (Dahl S rats) contains inhibitory factors that reduce the release of thromboxane A₂ from thrombin-activated platelets. Platelet-rich plasma from Dahl S rats on either low salt (0.11 or 0.3% NaCl) or high salt (4% NaCl) diets released about 50% less thromboxane A₂ than comparable plasma from Dahl rats that are resistant to hypertension (Dahl R rats). This inhibitory activity was present even in the blood of 4-week-old completely normotensive Dahl S rats on a diet containing 0.11% low NaCl. The inhibitory activity could be transmitted to platelets of normal Sprague-Dawley rats by incubating these platelets in boiled and dialyzed plasma from Dahl S rats. Moreover, the inhibitory activity could be completely washed off the Dahl S platelets by incubation in Dahl R plasma. Thus, Dahl S plasma contains inhibitory factors that reduce platelet thromboxane A₂ release. The factors are found in low concentrations even in Dahl R plasma; and in Dahl S or Dahl R plasma the factors are increased 25 to 32% by a 4% high NaCl diet. Digestion of Dahl S and Dahl R plasma with either trypsin or chymotrypsin destroyed the inhibitory factors, which have a molecular weight between 2000 and 3500. Twenty-four hours after bilateral nephrectomy, dialyzed plasma from both Dahl S and Dahl R rats was completely devoid of thromboxane A₂ inhibitory activity. Thus, the factors appear to be heat-stable polypeptides either produced in the kidney or greatly influenced by the presence of renal tissue. It is possible that these plasma factors somehow interact with kidneys, arterioles, or brain to make Dahl S rats highly susceptible to salt-induced hypertension. Moreover, these factors may constitute a genetic marker that could be clinically useful for recognizing the prehypertensive state in children.

(Hypertension 8 [Suppl II]: II-180-II-186, 1986)

KEY WORDS • juvenile hypertension • prostaglandins • plasma hypertensinogenic factors • hypertension prevention • genetic markers • kidney and hypertension

In a Dahl strain of rats genetically susceptible to salt-induced hypertension (Dahl S rats), the development of high blood pressure characteristicly depends upon the salt content in the diet.¹ The mechanism of salt-induced hypertension is still uncertain. However, there is evidence for facilitation of central and peripheral autonomic nervous discharge and retardation of the kidney's ability to excrete sodium.²⁻⁴ Reduced vasodilator prostaglandins (PGs) and increased vasoconstrictor PGs have also been found in the arterioles and quickly frozen kidneys of Dahl S rats.⁵ In addition, there is strong evidence that unusual constituents in their plasma may contribute to the development of high blood pressure in Dahl S rats.

Dahl and co-workers have shown that parabiotic Dahl S rats have hypertensinogenic substances in the circulating blood and, furthermore, that these substances may be derived from the kidney.⁶⁻⁷ The existence of hypertensinogenic substances in the plasma of Dahl S rats was confirmed by perfusion of the hindquarters.⁸ Injection of serum from Dahl S rats also evoked an elevation in blood pressure and an increased sensitivity of vascular pressor responses to vasoactive stimuli in bioassay rats.⁹ Moreover, an unusual component in the plasma of Dahl S rats has recently been detected with sodium dodecyl sulfate–polyacrylamide gel electrophoresis.¹⁰ The fraction was reported to be associated with the development of hypertension.

Thus, plasma from Dahl S rats contains unusual constituents that may be intertwined with the mechanism of salt-induced hypertension in this strain. However, research on these substances has advanced slow-
ly up to the present, mainly because sensitive assay methods have not been available. Hence, the search for any difference in plasma constituents between Dahl S rats and Dahl rats that are resistant to hypertension (Dahl R rats) may also reveal sensitive assay methods for detecting the hypertensinogenic substances.

We have already presented preliminary evidence that the release of thromboxane A₂ (TXA₂) from platelets is reduced in Dahl S rats, as compared with Dahl R rats. In the current study, we attempted to determine some of the characteristics of the plasma constituents in Dahl S rats that inhibit platelet TXA₂ release and to define the properties of these inhibitory factors.

Materials and Methods

Preparation of Platelet-Rich Plasma

Under anesthesia with methohexital sodium (Brevital Disodium, Eli Lilly, Indianapolis, IN, USA), Sprague-Dawley or Dahl rats were laparotomized, and 9 volumes of whole blood was drawn from the inferior vena cava into a syringe containing 1 volume of 3.8% Na₂ citrate. The anticoagulated blood was spun at 94 g at 25°C for 10 minutes. The supernatant platelet-rich plasma (PRP) was aspirated with a silicon-treated glass pipette. The PRP was used to measure the capacity to release TXA₂, according to the assay methods described below, or to obtain platelets for incubation with plasma samples from other rats, to examine their effect on platelet TXA₂ release.

Exchange of Plasma

To examine the effect of differences in plasma or medium on platelet TXA₂ release, platelets were incubated with the test plasma and then assayed according to the method described below. The prepared PRP derived from the anticoagulated whole blood of Sprague-Dawley or Dahl rats was spun at 550 g at 25°C for 2 minutes. The supernatant was discarded. The sedimented platelets were washed again and resuspended gently in the test plasma (or medium). The resuspended platelets were then incubated at 37°C for 30 minutes. Aliquots of this suspension were used for the following assay and for counting the number of platelets per volume of suspension.

Assay Method to Measure the Release of Thromboxane A₂ from Platelets

The PRP or the resuspended platelets were used to measure the capacity of platelets to release TXA₂. The assay mixture was composed of 500 µl of Dulbecco’s phosphate-buffered physiological salt solution at pH 7.35 (D-PBS, Gibco), 100 µl of a thrombin solution (10 U/ml), and 100 µl of the test PRP or resuspended platelet solution. The reaction was started with the addition of the thrombin solution to the assay mixture. The mixture was incubated at 37°C for 1 hour and then spun at 2000 g at 4°C for 10 minutes. The supernatant was stored at −70°C for use in the radioimmunoassay. TXA₂ released into the medium was directly radioimmunoassayed as thromboxane B₂ (TXB₂), using highly specific anti-TXB₂ antibodies. The concentration of TXA₂ in the assay mixture without the thrombin solution was subtracted from the measurement. The measurement was then related to the number of platelets in the assay mixture to determine the number of picograms of TXA₂ per 10⁶ platelets.

Radioimmunoassay

The radioimmunoassay was performed according to standard methods. Briefly, 0.1 ml of the sample diluted in buffer (1:200) or of the standard buffered TXB₂ solution, 0.1 ml of the diluted anti-TXB₂ antibody solution (1:200,000), and 0.1 ml of [¹H]TXB₂ (Amersham) solution (5000 cpm) were mixed and incubated at 4°C for 24 hours. After incubation, 0.1 ml of charcoal solution (2.5% charcoal and 0.25% dextran) was added to the ice-chilled assay mixture. The mixture was centrifuged at 2000 g at 4°C for 5 minutes. The supernatant was used to count [¹H]TXB₂ bound to antibody with a Beckman LS-330 scintillation counter.

The anti-TXB₂ antibody cross-reacted by 0.06% with PGD₂ and less than 0.01% with other PGs. The minimum detectable amount was 1.5 pg of TXB₂.

Blood Pressure Measurement

Blood pressure was measured by the tail cuff method of Friedman and Freed, with warming and without anesthesia in all rats over 4 weeks of age. A direct mean measurement of intracarotid blood pressure under Inactin anesthesia (80 mg/kg) was obtained in each 4-week-old rat. All Dahl rats came from Brookhaven Laboratories (Upton, NY, USA).

Statistical Analysis

The values are all expressed as means ± SE. The differences between means were analyzed by Student’s t test.

Results

Dahl R and Dahl S rats were fed a diet containing varying amounts of salt for 6 weeks after they had been weaned at 3 weeks of age. Dahl S rats on a very low (0.11%) NaCl diet remained normotensive, whereas Dahl S rats on a low (0.3%) NaCl diet or a high (4%) NaCl diet were borderline prehypertensive or hypertensive, respectively. No change in blood pressure was observed in Dahl R rats. As shown in Figure 1, in all three feeding groups, PRP from Dahl S rats had a markedly reduced capacity to release TXA₂, as compared with PRP from Dahl R rats. It was obvious that the suppressed release of TXA₂ from platelets of Dahl S rats was not related to the presence of high blood pressure, since the normotensive Dahl S rats also had reduced platelet TXA₂ release. The figure also shows that when Dahl rats were challenged with a high salt diet, platelet TXA₂ release significantly declined, both in Dahl R and in Dahl S rats.

A 66% reduction of platelet TXA₂ release was found even in very young (4-week-old) Dahl S rats on a 0.11% NaCl diet and with completely normal blood
pressure (Figure 2). Instead of utilizing PRP in these very small rats, we used a small amount of anticoagulated whole blood activated by thrombin according to the assay method. The removal of 0.18 ml of blood caused no change in blood pressure. The production of TXA₂ averaged 19 pg/10⁶ platelets for Dahl S rats, whereas the activated whole blood of Dahl R rats released 55 pg/10⁶ platelets.

Next, we attempted to elucidate the mechanism of the reduced platelet TXA₂ release in the PRP of Dahl S rats. We prepared plasma from Dahl R and Dahl S rats on a 0.11% NaCl diet for 6 weeks after weaning and tested the effect of the plasma on the release of TXA₂ from platelets of Sprague-Dawley rats. The plasma was boiled at 100°C for 10 minutes and centrifuged at 3000 g at 4°C for 30 minutes. The supernatant was dialyzed at 4°C in a membrane tube immersed in Dulbecco’s solution without Ca²⁺ and Mg²⁺ and then concentrated sixfold with dextran (molecular weight, 243,000). As depicted in Figure 3, platelets from Sprague-Dawley rats released 99.9 pg of TXA₂ per 10⁶ platelets when immersed in the treated Dahl R plasma but released only 10.3 pg of TXA₂ per 10⁶ platelets when immersed in the treated Dahl S plasma. Thus, by incubation of normal platelets from Sprague-Dawley rats in Dahl S plasma, the impaired platelet TXA₂ release could be transmitted to the normal platelets. In other words, plasma from Dahl S rats strikingly reduced the release of TXA₂ from normal platelets.

To verify this hypothesis, we exchanged the plasmas of PRPs between 9-week-old Dahl R rats and Dahl S rats, all on the 0.11% NaCl diet, and examined platelet TXA₂ release from the modified PRP. As shown on the left side of Figure 4, Dahl R rat platelets immersed in Dahl S rat plasma reduced platelet TXA₂ release by 47%, as compared with Dahl R rat platelets immersed in Dahl R rat plasma. The capacity of Dahl R rat platelets immersed in Dahl S rat plasma to release TXA₂ was virtually equal to that of Dahl S rat platelets immersed in Dahl S rat plasma. On the other hand, when Dahl S rat platelets were incubated in Dahl R rat plasma for 90 minutes, their ability to release TXA₂ was totally restored and equal to that of Dahl R platelets in Dahl R plasma (right side of Figure 4). These findings indicated that Dahl S plasma could transmit
Figure 3. Thromboxane A$_2$ (TXA$_2$) released from thrombin-activated platelets of Sprague-Dawley (SD) rats, preincubated in boiled, dialyzed, and concentrated plasma from Dahl salt-resistant (R) or salt-sensitive (S) rats. The open column shows SD platelets immersed in Dahl R plasma, and the shaded column shows SD platelets immersed in Dahl S plasma. The molecular weight cutoff included all molecules > 2000.

Figure 4. Effect of exchanged plasma on platelet thromboxane A$_2$ (TXA$_2$) release in platelet-rich plasma from Dahl salt-resistant (R) and salt-sensitive (S) rats. We prepared plasma from 16 Dahl R rats and from 16 Dahl S rats, all of which were 5 weeks old and had been fed a 0.11% NaCl diet for 2 weeks. An aliquot of plasma from each rat was digested with trypsin (5000 U/ml) or with chymotrypsin (800 U/ml). The inhibitory activity of the undigested and digested plasmas was examined (Figure 5 A and B). The undigested Dahl S rat plasma possessed enough inhibitory activity to reduce TXA$_2$ release from Sprague-Dawley platelets by ~29% to ~40%, as compared with Dahl R plasma. However, the digested Dahl S plasma completely lost the capacity to inhibit the release of TXA$_2$ from Sprague-Dawley platelets and had no more inhibitory activity than Dulbecco's salt solution. These results strongly suggest that the plasma from Dahl S rats did not lack the normal components for platelet TXA$_2$ release but instead contained a high concentration of inhibitory factors. Furthermore, these inhibitory factors included peptide bonds in their structure, which must be intact for the full inhibitory activity to occur. It should be pointed out that the digested Dahl R rat plasma also had a significantly reduced capacity to inhibit TXA$_2$ release from Sprague-Dawley platelets, as compared with the undigested Dahl R rat plasma.
This finding indicates that the inhibitory factors were present even in Dahl R rat plasma but at a concentration much lower than that in Dahl S rat plasma.

Next, we attempted to estimate the molecular weight of the plasma inhibitory factors, using molecular sieves. Pooled Dahl R plasma and pooled Dahl S plasma from 9-week-old rats on a 0.11% NaCl diet were both vigorously dialyzed against Dulbecco's salt solution without Ca\(^{2+}\) and Mg\(^{2+}\) at 4°C. The dialysis membranes used had specific molecular weight cutoff points. Without any dialysis, Dahl S rat plasma with its inhibitory factors induced a 40% lower release of TXA\(_2\) from Sprague-Dawley platelets, compared with Dahl R plasma (Figure 6). Dialysis membrane tubes with molecular weight cutoff points of 1000 or 2000 retained all the inhibitory activity of plasma from Dahl S rats. However, the tubes with a pore size of 3500 (molecular weight) apparently lost about half the inhibitory activity, and those tubes with a pore size larger than 15,000 (molecular weight) lost all the inhibitory activity. Consequently, we estimated the molecular weight of the inhibitory factors to be between 2000 and 3500.

We also assessed the influence of the kidney on the circulating inhibitory factors. Four-week-old Dahl R and Dahl S rats on a 0.11% NaCl diet underwent either a bilateral nephrectomy or a bilateral sham operation. Twenty-four hours after the operation, anticoagulated blood was collected and immediately centrifuged at 1000 g at 4°C for 30 minutes to obtain plasma. The plasma was then dialyzed against Dulbecco's salt solution without Ca\(^{2+}\) and Mg\(^{2+}\) at 4°C, with dialysis membrane tubes having a molecular weight cutoff point of 2000, in order to remove uremic toxins and concentrate the plasma twofold. The inhibitory activity in the plasma was tested according to the standard assay method. Whereas plasma from the Dahl S rats that underwent the sham operation produced a 30% reduction in TXA\(_2\) release from Sprague-Dawley platelets, as compared with plasma from Dahl R rats subjected to a sham operation, this difference was abolished when plasma from the nephrectomized Dahl R rats was compared with plasma from the nephrectomized Dahl S rats (Figure 7). Furthermore, the nephrectomy brought about a significant decrease in the plasma inhibitory activity with regard to the release of TXA\(_2\) from Sprague-Dawley platelets in plasma from both Dahl R and Dahl S rats. These findings indicate that nephrectomy somehow destroys the inhibitory activity in plasma from both Dahl strains. Our results suggest that the kidney either produces the inhibitory factors or has a strong influence on the level of or the inhibitory activity of these plasma factors.

**Discussion**

We demonstrated that Dahl S rats have a high concentration of inhibitory factors in circulating blood plasma that reduce the release of TXA\(_2\) from normal platelets. Moreover, the inhibitory activity was not brought about by the lack of some normal constituent in Dahl R rat plasma. On the basis of the results in the protease-treated plasma and in plasma from nephrectomized rats, the factors seemed to be present at a low
concentration even in plasma from Dahl R rats. However, the concentration of the factors was much lower in Dahl R rat plasma than in Dahl S rat plasma.

The inhibitory factors seemed to be partly influenced by the salt content in the diet. The high salt (4% NaCl) diet increased the inhibitory activity in plasma from both Dahl S and Dahl R rats, as compared with the very low salt (0.1%) NaCl diet. The high salt diet threatens Dahl S rats with a state of sodium retention, an integral component of this form of salt-induced hypertension. This tendency toward sodium retention induced by a high salt diet may have a physiological relationship to the inhibitory factors and may send signals that increase the concentration or activity of the factors in plasma.

These inhibitory factors were also found in the blood of very young normotensive Dahl S rats, which had normal blood pressure during the entire feeding period, even after blood had been drawn. Thus, it seems quite likely that the inhibitory factors are not secondary to rises in blood pressure but occur primarily as a genetic trait associated with a genetic predisposition for NaCl-induced hypertension.

The inhibitory factors were heat-stable and destroyed by trypsin or chymotrypsin. Thus, to express their full inhibitory activity, the factors appear to require an intact polypeptide in their molecular structure. The molecular weight was estimated to be between 2000 and 3500. Therefore, if the inhibitory factors consisted only of amino acids, the amino acid residues would number not more than 50.

Removal of the kidneys appeared to abolish the plasma inhibition of platelet TXA2 release. This observation indicates either that the factors were derived from the kidney or that renal tissue was required for the factors to have a high enough concentration and level of activity to exert an inhibitory effect on TXA2 release. Moreover, the inhibitory activity was completely gone 24 hours after the bilateral nephrectomy. This finding suggests that the factors are metabolized at a relatively fast rate in the circulation and have a rapid turnover rate.

The mechanism of the inhibition of platelet TXA2 release is uncertain. Besides TXA2 synthetase, PGD2 isomerase also occurs in the platelets. Using the method described by Patrono et al., we have examined the concentration of PGs in the serum of Dahl S and Dahl R rats before and after the clotting process has taken place. Although Dahl S rats had a significantly lower release of TXA2 than Dahl R rats, we found no difference in the release of PGD2 (unpublished data). Thus, it is conceivable that the inhibitory factors may specifically attack TXA2 synthetase in platelets rather than the earlier steps of PG metabolism, in which arachidonic acid is liberated from phospholipids and converted to the PG endoperoxides, PGG2, and PGH2. In these studies we have used only thrombin stimulation or natural clotting to stimulate TXA2 release from platelets. It is possible that the plasma inhibitory factors affect the thrombin-platelet interaction and have no direct effect on TXA2 synthesis.

Work is in progress to study the effect of the inhibitory factors on TXA2 release when platelet activators other than thrombin or clotting are used.

We do not have direct evidence that the inhibitory factors that reduce platelet TXA2 release can elevate blood pressure or sensitize vascular pressor responses to vasoactive stimuli. However, the inhibitory factors in this study have several similarities with hypertensinogenic substances described in previous studies. In those studies the substances were demonstrated only in Dahl S rats, not in Dahl R rats, and they appeared to come from the kidney of Dahl S rats or to be strongly influenced by it. The inhibitory factors that affect platelet TXA2 release were also profoundly influenced by the kidney of Dahl S rats, which apparently either produced or activated the factors. Similarly, the hypertensinogenic factors were demonstrable during a low NaCl diet in Dahl S rats, just as our platelet inhibitory factors were.

Despite these similarities, some discrepancies must be noted. The platelet inhibitory factors were detected even in Dahl S rats fed a low salt (0.11% NaCl) diet and also in Dahl R rats although at a much lower concentration. The hypertensinogenic factors described by Hirata et al. were found only in salt-fed hypertensive Dahl S rats. However, this discrepancy may be explained by a difference in the sensitivity of the assay methods employed. The method used to measure platelet TXA2 release may be more sensitive than the method used to measure blood pressure responses. Furthermore, even though the platelet inhibitory factors were abundant in Dahl S rats on a low NaCl diet, they were even more abundant in those on a high NaCl diet. Hence, a high NaCl intake in Dahl S rats increased the amounts of both the platelet inhibitory factors and hypertensinogenic factors, indicating an additional similarity between them.

Thus, the properties of the platelet inhibitory factors seem to suggest that these factors could be the same ones described in previous studies. It is conceivable that the platelet inhibitory factors affect the brain, the arterioles, and the kidney, predisposing Dahl S rats to NaCl-induced hypertension. Even in the prehypertensive state, the Dahl S rat is different from the Dahl R rat, as indicated by exaggerated central nervous system pressor responses, by shifted pressure natriuresis curves favoring sodium retention, and by increased concentrations of TXA2 and decreased concentrations of PGE2 and PG1 in low NaCl diet. A circulating humoral agent present in abnormally high concentrations in the Dahl S rats could account for some of these differences. When this humoral state is combined with a high NaCl intake, there could be marked responses in kidney, brain, and arterioles that bring about severe hypertension. The platelets are very reactive cells with many different receptors and their own contractile apparatus. It is quite feasible that the circulating platelets could be incidentally reacting to a humoral agent in plasma that is primarily concerned with regulating blood pressure, blood volume, and body sodium and is abundant in plasma from Dahl S rats.
Erne and co-workers\(^\text{17}\) have made such an observation with regard to the ionized calcium concentration in platelets in human essential hypertension. In our studies, the platelets could well have been affected as innocent bystanders by circulating humoral agents bent on sensitizing artery, brain, and kidney, and thus predisposing Dahl S rats to hypertension. Needless to say, however, this possibility cannot be tested directly until the inhibitory factors are purified and their physiological actions are ascertained.

Platelet inhibitory factors in plasma were markers of subsequent hypertension in our 4-week-old normotensive Dahl S rats. Similar factors in humans would be exceedingly useful for identifying children with a hereditary predisposition to develop hypertension as adults. It is conceivable that a platelet inhibitory factor in plasma could be used as a marker of future hypertension in children well before the development of high blood pressure, making it possible to institute appropriate preventive measures. This is only a hypothesis, however, since studies in humans have not yet been completed.

References

Platelet thromboxane inhibition by plasma polypeptides in prehypertensive Dahl rats.
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Hypertension. 1986;8:II180
doi: 10.1161/01.HYP.8.6_Pt_2.II180

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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