Vitamin E Did Not Prevent Platelet Activation, but Prevented Increase of Lipid Peroxides and Renal Dysfunction in DOCA-Salt Hypertensive Rats

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Summary Changes in platelet aggregability patterns and lipid peroxide levels and their relationship were examined in DOCA-salt hypertensive rats during the development of hypertension. In addition, the effect of vitamin E treatment on those changes was also investigated. The blood pressure of the hypertensive rats was 188, 201, 212 mmHg on day 11, 17, and 23, respectively. In the hypertensive rats, both aggregability and granule content in platelet decreased on day 12, 18, 24, and marked decreases were observed on day 24. These data suggested the appearance of exhausted platelets, which had been already activated in vivo due to the hypertension. Marked increases in lipid peroxide levels in serum, the heart and the kidney were observed on day 24 in the hypertensive rats. Increase in serum urea nitrogen was also observed in the hypertensive rats on day 24, suggesting the dysfunction of the kidney. Vitamin E treatment did not prevent in vivo platelet activation due to hypertension, but greatly prevented the elevations of lipid peroxides in serum, the heart and the kidney, and serum urea nitrogen. These results suggested that in vivo platelet activation, an increase of lipid peroxides, and renal dysfunction occur in this order due to hypertension, and that the latter two are significantly prevented by vitamin E treatment.

Key Words hypertension, platelet activation, vitamin E, lipid peroxide

Hypertension is known to be an important risk factor in atherosclerosis. On the other hand, both enhanced platelet function and oxidation of serum lipoprotein are known to play an important role in the initiation and maintenance of atherosclerosis. In hypertensive patients, enhanced platelet function and increase in serum lipid peroxide levels have been reported independently (1–6). Therefore, it would be of interest to know how platelet function and serum lipid peroxide levels change during the development of hypertension. However, little is known about the relationships between in vivo platelet activation and the elevation of serum lipid...
peroxide during the development of hypertension in either humans and animals.

It has been shown that vitamin E strongly prevents the oxidation of lipoproteins (7), and attenuates platelet functions such as aggregation, secretion, and adhesiveness to various surfaces (8-11). In addition, vitamin E has been reported to influence the production of prostacyclin (12), a potent inhibitor of platelet activity, and of the endothelial cell-derived relaxing factor, which also modifies platelet function (13,14). In fact, the increase of platelet aggregability, the reduced production of prostacyclin in vascular cells, and abnormal vascular relaxation have been shown in vitamin E-deficient animals (12,14). These facts suggest that vitamin E effectively prevents the enhancement of platelet function and the elevation of serum lipid peroxide levels, thereby improves the abnormal interaction between platelet and blood vessels due to hypertension.

We have observed that ADP- and collagen-induced platelet aggregation in whole blood decreased, and serum lipid peroxides increased in DOCA (deoxy-corticosterone acetate)-salt hypertensive rats in the malignant phase (15,16). The hypoaggregability of the platelets was accompanied by the reduction of granule content in platelet, indicating that the hypoaggregability was due to the presence of exhausted platelets which had already been activated in vivo. We have also observed that vitamin E did not prevent the appearance of exhausted platelets, but prevented the increase of serum lipid peroxides levels in the rats in the malignant phase (16).

In the previous studies (15,16), however, we could not define how platelet aggregability changes during the development of hypertension in the rats, and whether vitamin E prevents the platelets activation in vivo except in the malignant stage. We could not also define the relationship between platelet activation and the elevation of serum lipid peroxide. It was also unknown whether the increase of serum lipid peroxide, which has been shown to be cytotoxic (17,18), accompanied the increase of tissue lipid peroxide and tissue damage. In this study, therefore, we investigated the changes in platelet aggregability patterns and lipid peroxide levels in serum and organs, and the effect of vitamin E treatment of those changes during the development of hypertension in DOCA-salt hypertensive rats.

METHODS

1) Materials. Materials were obtained from the following sources: collagen (Hormon-Chemie, München GmbH, Germany), ADP (Sigma, St. Louis, MO), vitamin E (DL-α-tocopheryl acetate; Tokyo Kasei Kogyo, Tokyo, Japan), and DOCA (deoxy-corticosterone acetate; Wako Pure Chemical, Tokyo, Japan).

2) Experimental animals. Male Sprague-Dawley rats (about 150g) purchased from Japan Clea (Tokyo, Japan) were uninephrectomized and divided into 4 groups of 18 rats: normotensive (group A), normotensive treated with vitamin E (group B), DOCA-salt hypertensive (group C), and DOCA-salt hypertensive treated with vitamin E (group D). To induce DOCA-salt hypertension, the rats of
group C and D were injected with DOCA (15 mg/kg, s.c., twice a week) and simultaneously given 1% NaCl in place of water throughout the experimental period. The rats of group B and D received vitamin E (26 mg/rat, per os) dissolved in soybean oil every other day, and the rats of group A and C received an equivalent amount of soybean oil. Six rats from each group were sacrificed on day 12, 18, and 24.

3) Measurement of platelet aggregability. Blood was removed from the abdominal aorta under light anesthesia with ether. Since platelet functions are modified by other blood cells (19, 20), platelet aggregation test was performed by the single platelet counting method using whole blood to approximate in vivo situation (21). Whole blood (380 µl) anticoagulated with 1/10 (v/v) of 3.8% trisodium citrate was pipetted into a siliconized cuvette, while being stirred constantly at 1,100 rpm. After preincubation at 37°C for 3 min, 20 µl of ADP (10 µM) or collagen (100 µg/ml) was added. At 1 min after the addition of ADP or collagen, a 100 µl sample was taken into a tube containing 50 µl of 4.5% formaldehyde to fix platelet aggregates. The platelet count was measured with a particle counter (PC-601, Erma Optical Works, Japan) and the platelet aggregability was expressed by percentage decrease of a single platelet count as 100% of unstimulated platelets counts.

4) Measurement of serotonin content in platelets. Washed platelets prepared as described elsewhere (22) were suspended in a buffer (0.14 M NaCl, 15 mM Tris-HCl, 5 mM glucose, pH 7.4) to give 4 x 10^8 cells/ml. Serotonin content in platelets was measured according to Drummond and Gordon (23). The platelet suspension (0.2 ml) was mixed with 0.52 ml of 1.38 M trichloroacetic acid. The mixture was centrifuged for 5 min at 10,000 rpm. To 0.65 ml of the supernatant, 2.6 ml of the mixture of 1 volume of α-phthalaldehyde (0.5% in ethanol) and 10 volumes of 8 N HCl were added. Twenty-five microliters of potassium ferricyanide (0.2% w/v) were added to 0.65 ml of the supernatant to provide a tissue blank. The obtained mixture was heated for 10 min in boiling water to develop the fluorescence and the reaction was terminated by chilling. After nonspecific fluorescence was removed by mixing with 3 ml of CHCl₃, the intensity of fluorescence in aqueous phase was measured with a Hitachi Fluorescence Photometer (Hitachi Co. Ltd., Tokyo) at excitation and emission wavelengths of 360 and 475 nm, respectively.

5) Measurement of vitamin E. The vitamin E content in washed platelets, the heart and the kidney was analyzed by HPLC method as reported by Ueda and Igarashi (24). Briefly, 0.5 ml of platelet suspension or tissue homogenate was mixed with 0.3 ml of 2,2,5,7,8-pentamethyl-6-chromanol (an internal standard), and 0.3 ml of 6% pyrogallol in ethanol. Sixty microliters of 60% KOH were added to the mixture and it was saponified for 30 min at 70°C. After addition of 1.35 ml of 1% NaCl, vitamin E in the mixture was extracted with 4.5 ml of hexane containing 10% ethylacetate. The extract was evaporated under vacuum and dissolved in a 100 µl of hexane. Vitamin E in the extract was analyzed with a Hitachi HPLC system (L-2000) with a fluorimetric detector (F-1000).
6) Other measurements. Mean blood pressure was measured by the tail-cuff method in unanesthetized rats. Serum lipids (cholesterol, triglyceride, and phospholipids) were determined using kits from Kyowa Medics, Tokyo Japan. Serum urea nitrogen was assayed using diagnostic kits (Wako Chemical, Tokyo Japan). Lipid peroxides in serum and tissue were measured by the fluorometric method (25) and colorimetric method (26), respectively. Amount of protein was measured by the method of Lowry et al. (27).

7) Statistical analysis. Data presented are mean ± standard error (SEM). Statistical analysis was performed according to the Aspin-Welch test and significant difference with $p<0.05$ was used.

RESULTS

Changes in blood pressure and body, heart, and kidney weights

The blood pressure of the normotensive group (A) was maintained around 130 mmHg throughout the experimental period, while that of the hypertensive group (C) was markedly increased to the levels of 188, 201, 212 mmHg on day 11, 17, and 23, respectively (Fig. 1). Vitamin E treatment did not influence the blood pressure of the normotensive group (B), but slightly attenuated the increase in the blood pressure of the hypertensive group (D) on day 5 and 11. The vitamin E effect on the blood pressure in the hypertensive group (D) was not observed on day 17 and 23. Heart and kidney weights on day 12, 18, and 24 were significantly higher in the hypertensive groups (C and D) than in the normotensive groups (A and B) as

![Fig. 1. Changes in blood pressure. A and B: normotensive controls. C and D: DOCA-salt hypertensive. B and D: treated with vitamin E. Each point and vertical bar indicate the M±SEM for 5-6 rats. *h: significance ($p<0.05$, A vs C or B vs D), *e: significance ($p<0.05$, C vs D).](image-url)
shown in Table 1. Heart, kidney, and body weights were not influenced by vitamin E treatment either in the hypertensive group or in the normotensive group.

**Changes in platelet aggregability and the content of serotonin and vitamin E in platelets**

As shown in Fig. 2, platelet aggregation in whole blood induced by ADP and

| Treatment | Normotensive group | DOCA-salt hypertensive group |
|-----------|--------------------|-----------------------------|
|           | Vehicle (A) + Vitamin E (B) | Vehicle (C) + Vitamin E (D) |
| Body      |                    |                             |
| Day 12    | 250±5.32           | 248±7.07                    |
| Day 18    | 275±8.83           | 274±12.4                    |
| Day 24    | 281±8.27           | 304±13.6                    |
| Heart     |                    |                             |
| Day 12    | 0.94±0.013         | 0.99±0.030                  |
| Day 18    | 0.93±0.027         | 0.87±0.037                  |
| Day 24    | 0.97±0.028         | 0.96±0.042                  |
| Kidney    |                    |                             |
| Day 12    | 1.46±0.056         | 1.46±0.039                  |
| Day 18    | 1.56±0.055         | 1.56±0.074                  |
| Day 24    | 1.65±0.085         | 1.66±0.093                  |

Mean±SEM for 4–6 rats. *h: Significant change due to hypertension (A vs C or B vs D, p<0.05).

Fig. 2. Changes of ADP(a)- and collagen(b)-induced platelet aggregations in whole blood. Platelets in whole blood were stimulated by ADP (0.5 µM) or collagen (5 µg/ml) for 1 min and the aggregability was measured by the single platelet counting method. A and B: normotensive controls. C and D: DOCA-salt hypertensive. B and D: treated with vitamin E. Each vertical bar indicates the M±SEM for 5–6 rats. *h: significance (p<0.05, A vs C or B vs D). *e: significance (p<0.05, A vs B or C vs D).
collagen had already decreased by day 12 in the hypertensive group (C), and the change was marked on day 18 and 24, as compared with the normotensive group (A). The hypoaggregability observed in the hypertensive group (C) was accompanied by the decrease of platelet serotonin content (Fig. 3a), which is an indicator of the presence of exhausted platelets activated in vivo. These facts suggest that platelets from hypertensive group (C) had already become activated in vivo by day 12, and the activation had been marked by the sustained high blood pressure. Vitamin E content in platelets from the hypertensive group (C) on day 18 and 24 was also lower than those from the normotensive group (A) (Fig. 3b). Vitamin E treatment increased platelet vitamin E levels, and inhibited both ADP- and collagen-induced platelet aggregation in the normotensive group (B) (Fig. 2). However, the vitamin E treatment did not inhibit the decrease of serotonin content in platelets from hypertensive group (D) (Fig. 3a), suggesting that the vitamin E treatment did not prevent in vivo platelet activation due to hypertension.

Changes in lipids and lipid peroxides in serum and lipid peroxides and vitamin E in the heart and the kidney

Serum lipids in the hypertensive group (C) tended to increase on day 12 and significantly increased on day 24 (Fig. 4). Treatment with vitamin E tended to inhibit the increase of serum lipids in the hypertensive group (D). Lipid peroxide levels determined as thiobarbituric acid reacting substances values in serum, and the heart and the kidney increased in the hypertensive group (C), and the increase was marked in the kidney on day 24. Treatment with vitamin E increased vitamin E levels in the heart and the kidney (Fig. 6), and prevented the increases of lipid peroxides.
Fig. 4. Changes of the amount of cholesterol (a), triglyceride (b), and phospholipids (c) in serum. A and B: normotensive controls. C and D: DOCA-salt hypertensive. B and D: treated with vitamin E. Each vertical bar indicates the M±SEM for 5–6 rats. *h: significance (p<0.05, A vs C or B vs D).

Fig. 5. Changes of lipid peroxide levels in serum (a), heart (b), and kidney (c). A and B: normotensive controls. C and D: DOCA-salt hypertensive. B and D: treated with vitamin E. Each vertical bar indicates the M±SEM for 5–6 rats. *h: significance (p<0.05, A vs C or B vs D), *e: significance (p<0.05, A vs B or C vs D).
peroxide in serum, and the kidney of the hypertensive group (D) (Fig. 5). In contrast to platelets, no decrease of the vitamin E content in the heart and the kidney of the hypertensive group (C) was observed (Fig. 6).

Changes in serum urea nitrogen

To assess renal dysfunction, serum urea nitrogen was measured and shown in Fig. 7. The serum urea nitrogen in the hypertensive group (C) was increased on day 24. The treatment with vitamin E prevented the increase of serum urea nitrogen in the hypertensive group (D). These changes were consistent with those of lipid peroxide in serum and the kidney.

Fig. 6. Changes of \( \alpha \)-tocopherol content in the heart(a) and the kidney(b). A and B: normotensive controls. C and D: DOCA-salt hypertensive. B and D: treated with vitamin E. Each vertical bar indicates the \( M \pm SEM \) for 5-6 rats. *e: significance \( (p < 0.05, \ A \ vs \ B \ or \ C \ vs \ D) \).

Fig. 7. Change of serum urea nitrogen. A and B: normotensive controls. C and D: DOCA-salt hypertensive. B and D: treated with vitamin E. Each vertical bar indicates the \( M \pm SEM \) for 5-6 rats. *h: significance \( (p < 0.05, \ A \ vs \ C) \), *e: significance \( (p < 0.05, \ C \ vs \ D) \).
DISCUSSION

In this study, platelet aggregability test using whole blood was carried out to approximate the in vivo situation. The platelet aggregability in the hypertensive rats had already decreased by day 12, an early hypertensive stage, and the decrease had become marked with the high blood pressure sustained (Fig. 2). As reported previously (15, 16), hypoaggregability was accompanied by the reduction of serotonin content in platelets, indicating that the hypoaggregability was due to the presence of exhausted platelets which had already been activated in vivo. We have observed a similar phenomenon in spontaneous hypertensive rats (28, 29). However, the appearance of exhausted platelet in DOCA-salt hypertensive rats occurred earlier than that in spontaneous hypertensive rats. In the spontaneously hypertensive rats, more than 5 weeks of sustained high blood pressure was necessary to develop the exhausted platelets (28). This difference might be attributed to different rates in the development of hypertension. The faster the rate of development, the earlier the vascular injuries and exhausted platelets appear. As shown in Fig. 1, the development of hypertension in DOCA-salt-treated rats was so rapid that blood pressure above 180 mmHg was observed within 2 weeks after the treatment. DOCA-salt hypertensive rats have been shown to develop malignant vasculitis (30). In addition, it has been reported that rapid increase of blood pressure induced the damage of endothelial cells by oxygen radical (31). The damage of the endothelial cells, in turn, may enhance platelet adhesion to endothelial cells by decreasing the production of prostacyclin and the endothelial cell-derived relaxing factor, which have been reported to prevent platelet reactivity to the vascular wall (12, 13).

Increases of platelet aggregability in hypertensive patients have been reported in both in vitro (1, 2) and in vivo test (3–5). In contrast, we could not detect any increases of platelet aggregability throughout the development of hypertension in DOCA-salt hypertensive rats (Fig. 2). No increase of platelet aggregability and secretory response in in vitro tests was also observed either in experimental or spontaneous hypertensive rats, as reported previously (15, 21, 28, 29). The difference between humans and rats might be attributable to the different response to catecholamine in the platelets. Elevation of catecholamine in blood has been observed both in human and rat hypertension (32, 33). However, it has been shown that catecholamine induced aggregation and secretion of human platelets and intensified their responses to various other stimulants (34, 35), but did not induce the responses of rat platelets (36). Although our results in rats indicated that hypertension made platelets hypoaggregable in vitro, those data meant that platelet were hyperaggregable in vivo due to the hypertension. Therefore, the conclusion of our findings may be similar to that of the human studies in terms of the enhancement of platelets aggregability in vivo due to hypertension, which could result in the high incidence of thrombosis in coronary and cerebral arteries.
In DOCA-salt hypertensive rats, increases of lipid peroxide in serum, heart, and kidney were observed on day 24 when the blood pressure was sustained at more than 180 mmHg for 2 weeks. The marked elevation of lipid peroxide in the kidney of the hypertensive rat was accompanied by the increase of serum urea nitrogen, an indicator of renal dysfunction. Since lipid peroxide is known to be cytotoxic \((17, 18)\), renal dysfunction as assessed by the increase of serum urea nitrogen might be ascribed to the marked elevation of lipid peroxide in the tissue and blood. The mechanism underlying the increase of lipid peroxide in serum and tissue is not clear in the present study. However, the platelet activation \textit{in vivo} in the hypertensive rats could be partly involved in it. When platelets are activated, various lipid peroxides are formed and released from the platelets. We speculated that the lipid peroxides from the platelets consumed antioxidants in the blood, and increased lipid peroxide in serum and tissue. In fact, Chan \textit{et al.} \((37)\) have reported that 12-hydroperoxy-eicosatetraenoic acid, a lipoxygenase product in platelets, consumed vitamin E in cell free system. In the present experiment, platelet activation preceded the elevations of lipid peroxide by 2 weeks. These findings support our hypothesis.

Vitamin E administration inhibited platelet aggregability in normotensive rats. It was not clear from the aggregation data (Fig. 2) whether the vitamin E treatment also prevented platelet activation \textit{in vivo} in the hypertensive rats, because both vitamin E treatment and hypertension attenuated platelet aggregation \textit{in vitro}. On the other hand, it seems clear from the data of serotonin content in platelets that vitamin E administration did not prevent platelet activation \textit{in vivo} due to hypertension. No effect of vitamin E on the platelet activation may be associated with its slight antiaggregatory effect on platelet function, and with severe vascular injuries which might greatly activate platelet \textit{in vivo}. It has been reported that the effect of vitamin E on platelet function is weak, especially in \textit{in vivo} situation \((9, 10)\), and that DOCA-salt hypertensive rats develop malignant vasculitis \((30)\).

It is well known that vitamin E is a potent antioxidant and prevents lipid peroxidation in lipoprotein and cell membrane \((7)\). In the present experiment, the treatment with vitamin E significantly prevented the increases of lipid peroxide levels in various tissues, and serum urea nitrogen. It has been reported that ischemia and sequent reperfusion system generates oxygen free radicals and increases lipid peroxide in the tissues, and the increases of lipid peroxide and tissue damage were prevented by vitamin E treatment \((38, 39)\). Our present result regarding the vitamin E effect on lipid peroxides and serum urea nitrogen is consistent with those reports.

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