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Increase in number of Weibel-Palade bodies and endothelin-1 release from endothelial cells in the cadmium-treated rat thoracic aorta

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Abstract Male rats received daily intraperitoneal injections of cadmium sulphate (2.0 mg/kg) for 3 (Cd-3 group), 6 (Cd-6 group) and 8 days (Cd-8 group). The blood samples were prepared for endothelin (ET)-1 assay, and the thoracic aorta was investigated by both electron microscopy and immunoelectron microscopy using anti ET-1 sera. The plasma ET-1 concentrations of both Cd-6 and Cd-8 groups increased significantly in a cumulative dose-dependent manner. The cadmium-treated rat aorta showed an increase in the number of Weibel-Palade (WP) bodies in endothelial cells, and degranulation and exocytosis of WP bodies occurred exclusively in the Cd-8 group. Immunoreaction for ET-1 was localized preferentially in WP bodies of both cadmium-treated and control groups, and in the rough endoplasmic reticulum of the cadmium-treated groups only. Reactivity was also found on the WP bodies undergoing exocytosis in the Cd-8 group. Cadmium intoxication induces an increase in number of ET-1-storing WP bodies in the rat aorta endothelium. The enhancement of extracellular release of their contents by exocytosis results in elevation of the plasma ET-1 concentration.

Key words Cadmium toxicity · Endothelin · Immunoelectron microscopy · Plasma endothelin concentration · Weibel-Palade body

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Introduction

Cadmium, a heavy metal trace element, is now widely used in many industries and has a broad range of toxic effects. Cadmium has been reported to induce hypertension in vivo [6, 32] and in vitro [22, 25], vasoconstriction in vitro [7, 23] and atherosclerosis in vivo [26]. These findings suggest that cadmium provokes primarily arterial injury. Nolan and Shaikh [24] supposed that the initial effect of acute cadmium administration was on the integrity and permeability of the vascular endothelium and that various necrotic changes occurred secondarily in the subendothelial layer. As various endothelial cell injuries are risk factors in cardiovascular diseases [27, 28], it is reasonable to assume that the endothelial cell injuries induced by cadmium intoxication may trigger such diseases.

The toxic effects of cadmium salts on the rat thoracic aorta were revealed by X-ray microanalysis combined with electron microscopy [42]. The report on this study described the accumulation of cadmium in the mitochondria and the increase in number of Weibel-Palade (WP) bodies in the endothelial cells, but no quantitative analyses as to the ultrastructural changes of the WP bodies were done. Since Weibel and Palade [39] first described WP bodies in small rat arteries, the function of these inclusions has been analysed by many researchers. Recently, it has been revealed that WP bodies are a storage site of the von Willebrand factor [12, 37] and GMP-140, a platelet α-granule membrane protein [2, 16]. Bertini and Santolaya [1] suggested a hypertensive activity for WP bodies in the toad aorta. A drastic increase in number of WP bodies occurred in the rabbit umbilical vein during the perinatal stage, correlating with an elevation in histamine concentration determined by high-performance liquid chromatography [9]. Furthermore, our recent immunocytochemical studies revealed that endothelin (ET)-1, which possesses a potent vasoconstrictive activity [41], was stored in WP bodies [30].

ET-1 synthesis is enhanced in injured endothelial cells [19, 34]. Elevation of plasma ET-1 concentrations has

been reported in patients with essential hypertension [13, 29], coronary vasospasm [36] and cerebral vasospasm [8, 33]. These data suggest that the elevation of the plasma ET-1 level may reflect endothelial cell damage. However, to date, no ultrastructural analyses after acute cadmium intoxication have been combined with both immunocytochemistry of ET-1 and assay for the plasma ET-1 concentration.

This study was designed to investigate whether an increase in the number of WP bodies can be evaluated by quantitative analyses, whether this increase is proportional to the elevation of the plasma ET-1 level, and whether ET-1 stored in the WP bodies shows dynamic changes such as were revealed by immunocytochemistry in the cadmium-treated rat thoracic aorta.

Materials and methods

Animals

Male Wistar rats aged 8 weeks and weighing 250±10 g were housed in individual stainless steel cages and maintained under a 12-h light-dark cycle. The rats were fed laboratory chow and water ad libitum. In the care and use of the animals utilized for this study, the authors followed the Guiding Principles for the Care and Use of Animals approved by the University of Occupational and Environmental Health in accordance with the principles of the declaration of Helsinki (1964, revised in 1975, 1983).

Tissue preparation

In our preliminary study, we determined that 8.0 mg/kg body weight of cadmium sulphate ($CdSO_4$) i.p. daily for 8 days was the LD_{50} (lethal dose for the 50% survival group) in the male Wistar rats. A quarter of the LD_{50} was chosen as the dose to be used in the present study.

The rats (four rats per group) were injected daily i.p. with 2.0 mg/kg body weight of $CdSO_4$ for 3, 6, and 8 days. (The groups are referred to as Cd-3, Cd-6 and Cd-8, respectively, in this text.) The control group (four rats) received an equivalent volume of saline daily for 8 days. Twenty-four hours after the last injection, animals were deeply anaesthetized with an injection of pentobarbital and perfused with physiological saline through the left ventricle for 5 min at 37°C, followed by a solution of periodate–lysine paraformaldehyde [17] (PLP, 0.0375 M phosphate buffer containing 0.01 M NIO_4, 0.075 M lysine and 2% paraformaldehyde) for 10 min at 4°C. Prior to perfusion, blood samples for the endothelin (ET)-1 assay were collected from the left ventricle.

For electron microscopy, the thoracic aortae were immersed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4°C and postfixed in 1% osmium tetroxide in the buffer for 2 h at 4°C. After dehydration in graded concentrations of acetone, specimens were embedded in epoxy resin. Ultrathin sections were stained with 5% uranylacetate for 6 min and lead citrate for 4 min, and then examined in a JEM 1210 electron microscope.

For immunoelectron microscopy, the vessels of each group were immersed in a solution of the PLP for 6 h at 4°C, dehydrated in graded concentrations of ethanol, and embedded in epoxy resin. Ultrathin sections were mounted on uncoated 150-mesh nickel grids and placed for 15 min on drops of 1.0% egg albumin in PBS (EA-PBS) to absorb non-specific proteins. The sections were then incubated for 2 h with mouse anti ET-1 monoclonal antibody against the N-terminal of ET-1 (Yamasashoyu, Chiba, Japan) diluted at 1:1000 in 0.1% EA-PBS. After rinsing in PBS, the grids were reacted to goat anti-mouse IgG-coated 15 nm colloidal gold (Ultra Biosols, Liverpool, UK) diluted at 1:100 in 0.1% EA-PBS for 1 h, washed in PBS, and then washed in distilled water. Sections were stained with 5% uranyl acetate only for 3 min and examined in the electron microscope.

The specificity of the immunolabellings was confirmed by replacing the primary antibody with either normal mouse serum or PBS.

Plasma ET-1 quantification

Blood samples (4 ml each) were immediately centrifuged at 3,000 rpm for 10 min, and stored at –20°C until assay for ET-1 in polypropylene tubes containing a final concentration of 300 KIU/ml of aprotinin (Wako, Osaka, Japan) and 2 mg/ml of EDTA. ET-1 was extracted from the plasma (0.5 ml) using Seppak C-18 cartridges (Waters, Milford, Mass.).

The assay for the ET-1 concentration was carried out according to the sandwich-EIA method [35] using the ET-1 EIA kit (Wako, Osaka, Japan) and measured by a microplate reader system (MPA-9300, Shimadzu, Kyoto, Japan) equipped with a spectro-fluorometer (492 nm). For statistical comparisons between data from the control group and data from the cadmium-treated groups, Student's *t*-test for unpaired comparisons was used.

Quantitative evaluation of Weibel-Palade bodies

Quantitative analyses on the number and the area of WP bodies per 100 μm^2 endothelial cells area were carried out with an image-analysing device (Nikon Cosmosone 1S). The numbers of swollen and exocytosed WP bodies were also analysed by this device: 30 electron micrographs of randomly selected endothelial cells from 4 vessels of each group were taken at ×20,000 and printed at a final magnification of ×40,000. WP bodies with a normal appearance and altered WP bodies were counted directly in the electron micrographs, and the area of each endothelial cell and total area occupied by the WP bodies per cell were measured. These data are shown in Table 1 (mean±SEM). For stastical comparisons between data from the control group and data from the cadmium-treated group, Student's *t*-test for unpaired comparisons was used.

Table 1 Quantification (mean±SEM) of Weibel-Palade (WP) bodies in rat thoracic aorta by means of image analysis (*EC* endothelial cell)

	Number of WP bodies per 100 μm^2 EC area	Total area of WP bodies (μm^2) per 100 μm^2 EC area	Number of altered WP bodies per 100 μm^2 EC area
Control group (30 cells from 4 vessels)	113.4±10.6	1.17±0.10	1.61±0.82
Cd-3 group (30 cells from 4 vessels)	190.2±10.0*	2.40±0.15*	4.17±1.68
Cd-6 group (30 cells from 4 vessels)	350.7±15.5*	4.61±0.20*	7.82±3.42
Cd-8 group (30 cells from 4 vessels)	230.2±19.7*	3.21±0.28*	40.76±3.11*

* $P<0.01$

Results

Plasma ET-1 concentrations

Figure 1 shows plasma ET-1 concentrations of the control and cadmium-treated (Cd-3, Cd-6 and Cd-8) groups, and the increase in the mean plasma ET-1 concentration is seen to have occurred in a cumulative dose-dependent manner. The mean plasma ET-1 concentration of the Cd-6 and Cd-8 groups increased significantly compared with that of the control group ($P<0.05$ and $P<0.01$, respectively), while there were no significant differences between that in the Cd-3 group and that in the control group.

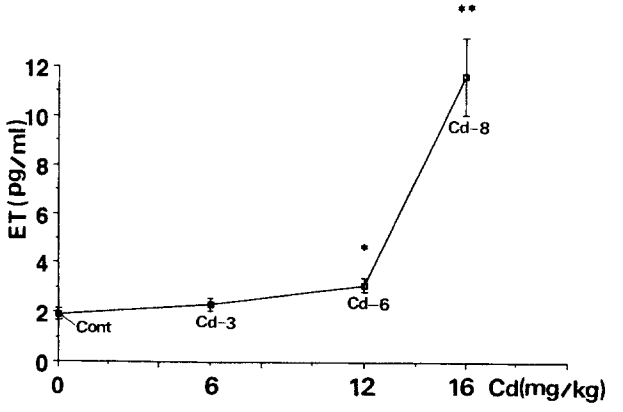

Fig. 1 Mean plasma ET-1 concentrations (ET) in the control (*Cont*) and the cadmium-treated groups (*Cd-3*, *Cd-6* and *Cd-8*). The increase in the plasma ET-1 concentration occurs in a cumulative and dose-dependent manner (*Cd* total dose of $CdSO_4$, mg/kg). *Vertical bars* indicate SEM; *asterisks* show statistically significant differences between the control and cadmium-treated groups (★, $P<0.05$; ★★, $P<0.01$)

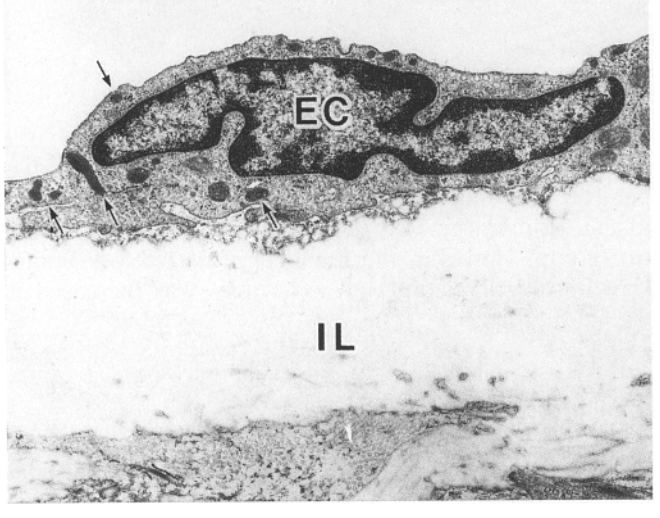

Fig. 2 An endothelial cell (*EC*) in a thoracic aorta from the control group contains a considerable number of WP bodies (*arrows*) (*IL* internal elastic lamina; ×13,000)

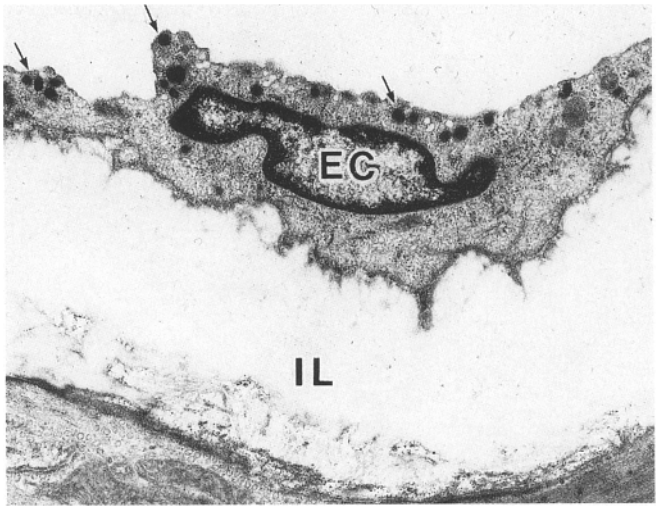

Fig. 3 Increase in the number of WP bodies occurs in the Cd-3 group (*arrows*), and most of them aggregate near the apical plasma membrane (*EC* endothelial cell; *IL* internal elastic lamina; ×13,000)

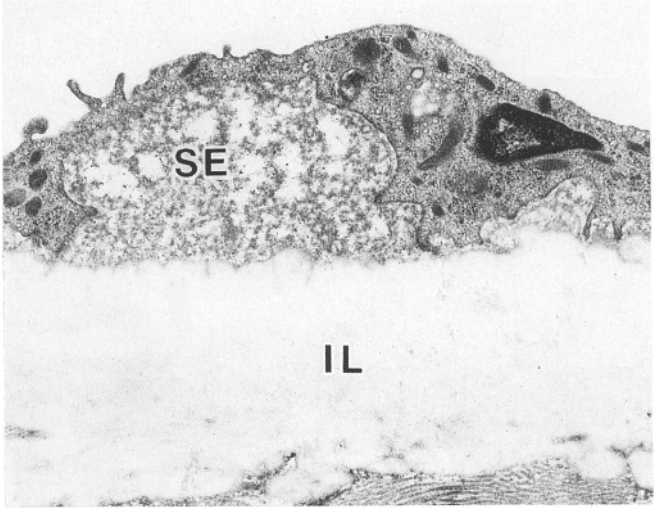

Fig. 4 The subendothelial space (*SE*) becomes severely oedematous in the Cd-6 group. (*IL* internal elastic lamina; ×10,000)

Cytological observations

Endothelial cells of the thoracic aorta in the control group contained a considerable number of WP bodies, and i.p. injection of saline alone did not result in any significant ultrastructural changes, such as swelling and degranulation of the WP bodies (Fig. 2). Aggregation of WP bodies near the apical plasma membrane of the endothelial cells sometimes occurred in the cadmium-treated samples from the Cd-3 group, as shown in Fig. 3. The subendothelial layer became severely oedematous in the Cd-6 group (Fig. 4).

An increase in the number of WP bodies was first evident in the Cd-3 group and was much more pronounced in the Cd-6 (Fig. 4) and Cd-8 groups (Fig. 5). Lysosomal

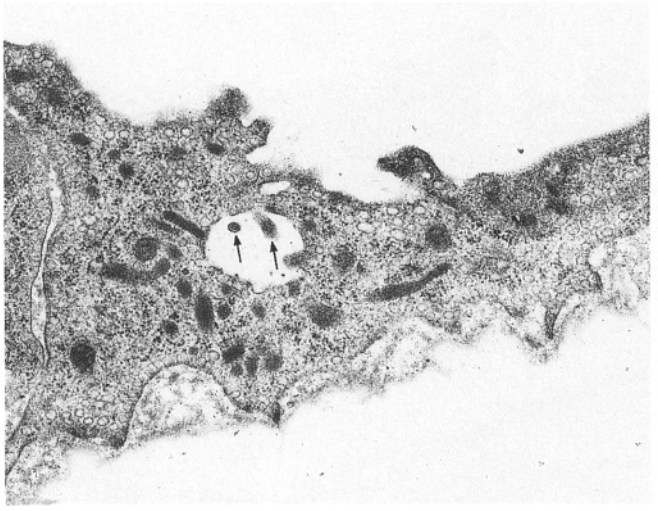

Fig. 5 A remarkable increase in the number of WP bodies occurs in an endothelial cell in the Cd-8 group. A lysosomal vacuole includes remnants of WP bodies (*arrows*). (×17,000)

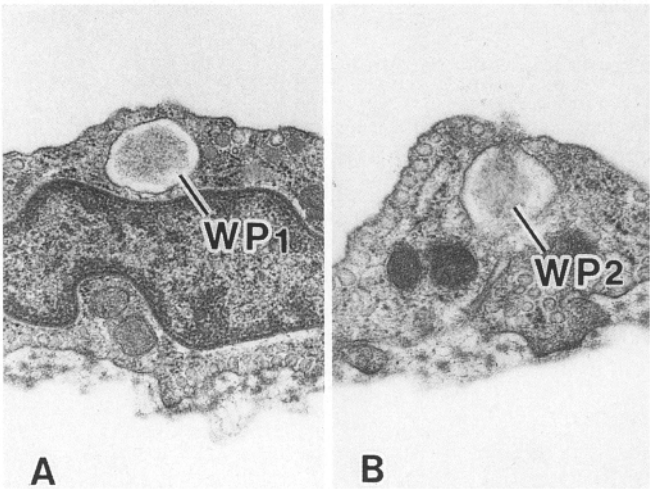

Fig. 7A, B Degranulation of a WP body (WP_1) and exocytosis of a WP body (WP_2) from the apical plasma membrane are shown in endothelial cells in the Cd-8 group. (**A** ×25,000; **B** ×25,000)

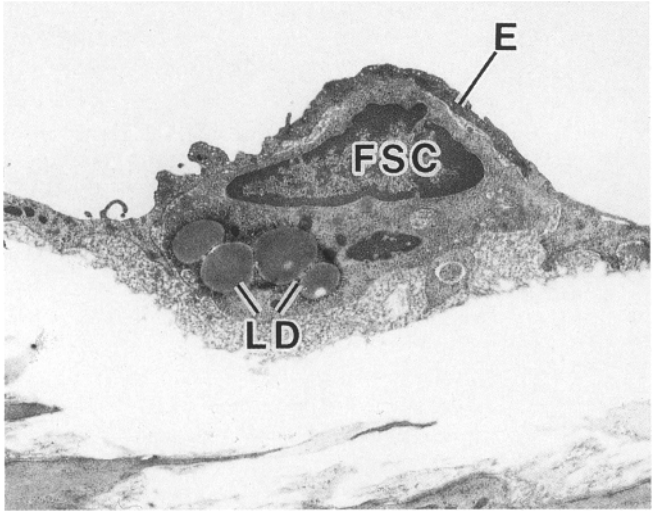

Fig. 6 A fat-storing cell (*FSC*) exists below the endothelium (*E*) in the Cd-8 group. (*LD* lipid droplets; ×6,000)

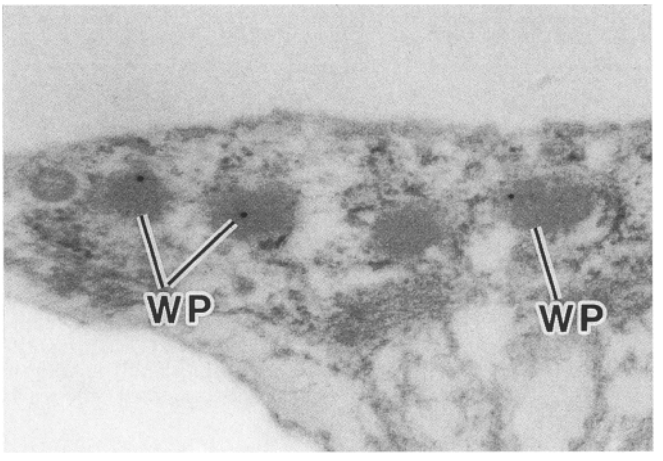

Fig. 8 Immunoreactive gold particles of ET-1 are preferentially localized on WP bodies (*WP*) in the control group. (×45,000)

vacuoles occasionally included remnants of the WP bodies in the Cd-8 group (Fig. 5). Fibroblast-like cells containing numerous lipid droplets, which are considered to be fat-storing cells, were occasionally found below the endothelium in the Cd-8 group (Fig. 6).

Ultrastructural alterations of the WP bodies were pronounced in the Cd-8 group: these included decrease in electron density, swelling with a wide peripheral halo between an osmiophilic dense core and the limiting membrane (Fig. 7A) and extrusion of the contents by exocytosis from the apical plasma membrane (Fig. 7B).

Quantitative determination of WP bodies

The quantitative data are summarized in Table 1: the number of WP bodies and the total area of WP bodies per 100 μm² endothelial cell area increased significantly in the cadmium-treated groups compared with the control group. However, the number of altered WP bodies showing degranulation and exocytosis was increased in the Cd-8 group only.

Immunoelectron microscopy

In the control group, ET-1-immunoreactive gold particles were localized on some WP bodies (Fig. 8), but few were found on the rough endoplasmic reticulum (rER) and Golgi apparatus. In contrast, we often encountered immunoreactions on the rER in all the cadmium-treated

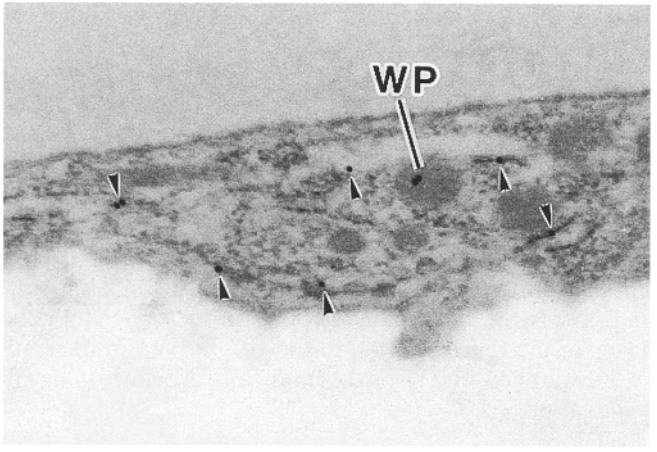

Fig. 9 The immunoreactive gold particles are localized in cisterns of the rER (*arrowheads*) and on a WP body (*WP*) in the Cd-3 group. (×45,000)

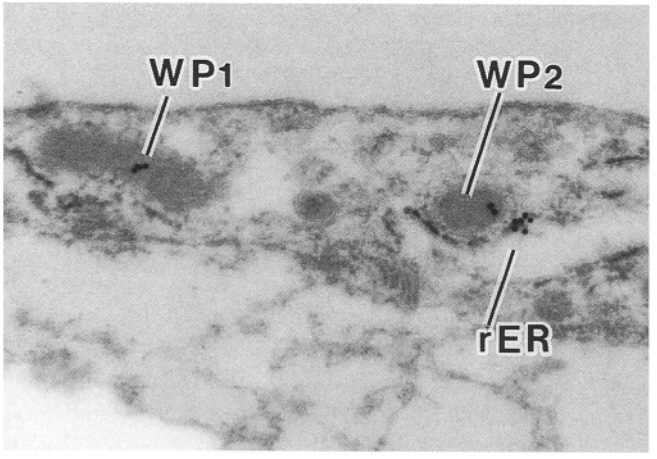

Fig. 10 The immunoreactive gold particles are localized on WP bodies (WP_1 and WP_2) and in a dilated cistern of the rER closely apposed to a WP body (WP_2) in the Cd-6 group. (×45,000)

groups (Fig. 9). The immunoreactions both on WP bodies and on rER became more pronounced in both the Cd-6 and Cd-8 groups than in the Cd-3 group: in the Cd-6 and Cd-8 groups, the dilated rER, which was closely associated with the WP bodies, showed intense immunoreaction for ET-1 (Fig. 10).

In the Cd-8 group, the degranulation and exocytosis of the WP bodies from the apical plasma membrane was pronounced. The immunoreactive gold particles were often localized in the apical part of the endothelial cytoplasm near the degranulated WP bodies (Fig. 11A), as well as on the WP bodies undergoing exocytosis (Fig. 11B).

Little or no immunoreactivity was observed when the primary antibody was replaced by either normal mouse serum or PBS for negative controls.

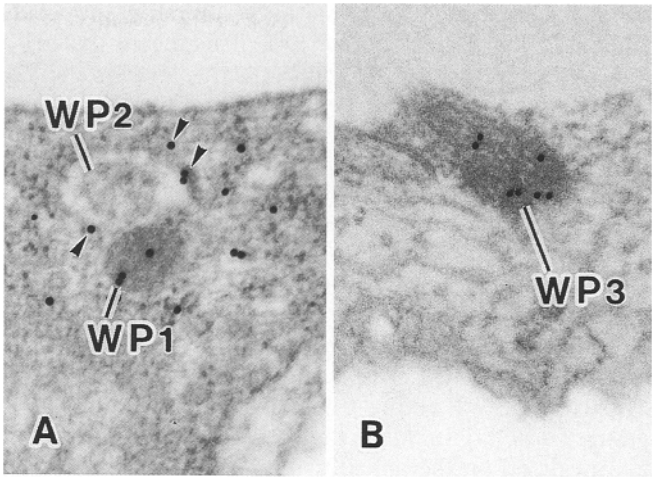

Fig. 11 **A** The immunoreactive gold particles are localized on a WP body (WP_1) and in the cytoplasm (*arrowheads*) around a swollen WP body (WP_2) in the Cd-8 group. (×45,000). **B** The immunoreactive gold particles are localized on a WP body (WP_3) that is in contact with the apical plasma membrane of an endothelial cell in the Cd-8 group. (×45,000)

Discussion

In the present study, significant ultrastructural changes occurred in the endothelial cells of the cadmium-treated rat thoracic aortae. These included increase in number of WP bodies and degranulation of these inclusions. Similar cadmium-induced changes have already been demonstrated in the thoracic aortae of pregnant rats after a much lower dose (9.0 mg/kg total dose of $CdSO_4$) [42] than our dose (16.0 mg/kg total dose of $CdSO_4$ in the Cd-8 group). Samarawickrama and Webb [31] reported pathological changes in the liver and kidney following acute cadmium intoxication in pregnant rats only. The present ultrastructural data are the first to describe cadmium-induced endothelial cell injuries in non-pregnant rats and to support the suggestion of Samarawickrama and Webb that quite different doses are needed in pregnant than in non-pregnant animals for the induction of cadmium intoxication.

In addition to these ultrastructural changes in the endothelial cells, oedematous changes in the subendothelial layer were observed in the cadmium-treated rat aortae. Since endothelial cells act as a permeability barrier between blood and the underlying connective tissue, injury to endothelial cells may disturb this barrier function [27].

Increase in the number of WP bodies in endothelial cells was the major morphological change in the Cd-3 group. Recently, it has been revealed that WP bodies are a storage site for ET-1 [30]. Since Yanagisawa et al. [40] purified ET-1 from the medium of cultured endothelial cells, pharmacological and physiological analyses have elucidated its potent vasoconstrictive activity [41]. Our previous study on the immunolocalization of ET-1 in endothelial cells of the perinatal rabbit umbilical vein showed that ET-1-immunoreactive gold particles are lo-

calized preferentially in cisterns of the rER and Golgi apparatus, and on WP bodies [30]. This means that ET-1 is synthesized in the rER Golgi system, stored in the WP bodies, and released extracellularly from the WP bodies, although this consideration argues against the constitutive pathway theory proposed by Nakamura [21]. In the present study, immunoreactions for ET-1 were also found on the WP bodies in the control group – WP bodies are also involved in the storage of ET-1 under normal conditions. However, the dilatation of the rER and the immunoreactivity of ET-1 on such dilated cisterns, which are closely apposed to the WP bodies, were found exclusively in the Cd-6 and Cd-8 groups. Degranulation and exocytosis of the WP bodies frequently occurred in the Cd-8 group, and ET-1-immunoreactive gold particles were often localized on the apical cytoplasm near the degranulated WP bodies and on the WP bodies under exocytosis in the Cd-8 group. These results indicate that the enhancement of ET-1 synthesis by the rER, ET-1 storage in the WP bodies, and ET-1 discharge from the WP bodies into the blood occurred following administration of cadmium salts in a dose-dependent manner. Since the activation of ET-1 synthesis also occurred in the injured endothelial cells with an endotoxin [34] and polymorphonuclear leukocytes [19], further quantitative analyses on the expression of ET-1 messenger RNA in cadmium-treated and non-cadmium-treated rats are necessary.

The results of the present study show that the increase in the plasma ET-1 concentration is proportional to the increase in the number of WP bodies. The highest plasma ET-1 concentration was assayed in the animals in group Cd-8, whose WP bodies showed predominantly degranulation and exocytosis. Since it is widely accepted that the elevation of the plasma ET-1 level depends in part on endothelial cell damage [18, 34], the cadmium toxicity against endothelial cells in the samples may be a crucial causative factor in the rise of plasma ET-1 concentration. We have reported that ET-1 evokes the degranulation and exocytosis of WP bodies [4, 5] and we now consider that the ET-1 released from the injured endothelial cells may act on the endothelial cells themselves in an autocrine manner, which may in turn induce the synergistic excretion of the ET-1 mediated by WP bodies. However, we cannot deny the possibility that an overflow of ET-1 may occur in the injured endothelial cells that have broken down [10, 11]. In order to reveal the exact pathway of ET-1 synthesis and release in the cadmium-treated endothelial cells, further studies to compare the immunolocalization of big-ET-1, a prosequence peptide of ET-1 [41], and the plasma concentration of big-ET-1 with the immunolocalization and plasma concentration of ET-1 are necessary, and these are now in progress in our laboratory.

Cadmium is a potent nephrotoxin, which causes significant damage to the proximal tubular epithelium [20]. A recent report showed that the renal glomerulus is also targetted by cadmium [3]. Since ET-1 mediates drug-induced renal vasoconstriction and glomerular dysfunction [14, 15], we now consider that ET-1 may play an important part in fostering progression of the cadmium-induced renal dysfunction. Thus, it is necessary to solve the problem as to whether an acute elevation of plasma ET-1 concentration induced by the endothelial injury in our samples results in subsequent renal dysfunction.

The appearance of fat-storing cells below the endothelium has been reported in pancreatic [38] and uterine [43] blood vessels after cadmium intoxication. The involvement of these cells in a defence function against cadmium toxicity has been reported [38]. Since fat-storing cells were occasionally found in the subendothelial layer of the thoracic aorta in the Cd-8 group in the samples examined, the roles of these cells are now under investigation.

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