Pflügers Arch (1987) 408:479–483

Pflügers Archiv
European Journal of Physiology

Effects of glutaraldehyde fixation on renal tubular function

I. Preservation of vasopressin-stimulated water and urea pathways in rat papillary collecting duct

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Abstract. Using the in vitro microperfusion technique on isolated rat papillary collecting duct (PCD), we examined whether the glutaraldehyde-fixation method can be also applied to the mammalian collecting duct for preservation of the vasopressin-stimulated water and urea transport. Arginine vasopressin (AVP) at 10^{-9} mol/l increased diffusional water permeability (P_{dw}) from 101.9 ± 10.76 to $283.3 \pm 16.67 \times 10^{-7}$ cm^2 s^{-1} ($n = 8$, $P < 0.01$) and urea permeability (P_{urea}) from 30.3 ± 2.24 to $83.5 \pm 7.80 \times 10^{-7}$ cm^2 s^{-1} ($n = 8$, $P < 0.01$). Both parameters remained elevated after fixation with 0.1 mol/l glutaraldehyde even in the absence of AVP, with the values being 265.0 ± 14.47 and $74.5 \pm 7.15 \times 10^{-7}$ cm^2 s^{-1}, respectively. Glutaraldehyde fixation did not affect the basal levels of P_{dw} or P_{urea}. Phloretin at 2.5×10^{-4} mol/l decreased glutaraldehyde-fixed AVP-stimulated P_{urea} from 79.0 ± 7.96 to $29.7 \pm 3.66 \times 10^{-7}$ cm^2 s^{-1} ($n = 4$, $P < 0.01$) and from 73.2 ± 7.05 to $38.7 \pm 3.53 \times 10^{-7}$ cm^2 s^{-1} ($n = 4$, $P < 0.01$) when the drug was added to the lumen or to the bath, respectively. Phloretin also decreased glutaraldehyde-fixed non-stimulated P_{urea} by 25–40%. However, this drug did not affect glutaraldehyde-fixed P_{dw}. These findings indicate that the glutaraldehyde fixation method can be applied to mammalian collecting tubules for studying vasopressin stimulated P_{dw} and P_{urea}. P_{urea} fixed by glutaraldehyde is functionally flexible and may be distinct from the water pathway.

Key words: Papillary collecting duct – Vasopressin – Phloretin – Water channel – Urea channel – Glutaraldehyde

Introduction

It has been well established that antidiuretic hormone increases both water and urea permeabilities. Extensive studies on the urinary bladder [2, 3, 26] has demonstrated that vasopressin-stimulated water channels are clearly distinct from the urea channels. However, this has not been fully confirmed in the mammalian kidney. The indirect evidence in support of this view in mammalian kidney is as follows: vasopressin increases only water permeability in the cortical and outer medullary collecting tubule [25], whereas it increases both water and urea permeability in the rat papillary collecting duct [18, 24]. But it has not yet been established whether the vasopressin-stimulated water permeability in the rat papillary collecting duct is distinct from urea permeability. Therefore, the major purpose of the present study was to examine this issue, using isolated perfused rat papillary collecting ducts. For this purpose, we specifically examined whether phloretin selectively inhibits vasopressin-stimulated urea permeability.

Convincing evidence has accumulated in support of the hypothesis that the vasopressin-stimulated water permeability represents a change in a dynamic state of equilibrium between the insertion and extrusion of water-channel molecules from cytoplasm into cell membranes [19, 27]. If this is also the case for urea permeability, it is difficult to assess whether a transport inhibitor exerts its action directly on channels or on the process of channel insertion. In 1972, Eggena [6, 7] reported that glutaraldehyde treatment of the toad urinary bladder can preserve vasopressin-stimulated water permeability. This procedure has the advantage that it allows the vasopressin-stimulated water channels to be studied under stable conditions [6–10, 12–14, 20, 21]. It has also been reported that in the amphibian urinary bladder, vasopressin-stimulated urea permeability is preserved by glutaraldehyde treatment [12, 21].

Since it has not been confirmed whether the glutaraldehyde fixation method is also applicable to the mammalian renal tubules, we first tested this issue. Then we applied the glutaraldehyde fixation method to the study of discriminating between water and urea channels. The results indicate that both vasopressin-stimulated water and urea permeabilities are well preserved by the glutaraldehyde treatment, and that the urea permeability is selectively inhibited by phloretin even after glutaraldehyde fixation.

Materials and methods

Male Wistar rats weighing about 200–350 g were decapitated with a guillotine and the both kidneys were removed to make slices. Kidney slices of about 1 mm thickness were placed in a dish containing an artificial solution in mmol/l; NaCl 110, $NaHCO_3$ 25, KCl 5, Na_2HPO_4 1.6, NaH_2PO_4 0.4, Na acetate 10, $MgCl_2$ 1.0, $CaCl_2$ 1.8, D-glucose 8,3, and L-alanine 5. Papillary collecting duct (PCD) from the mid-papilla was isolated with sharpened fine forceps under a stereomicroscope.

Isolated renal tubules were perfused in vitro in a bath at 37° C according to the method of Burg et al. [1] with slight modifications. A light microscopic picture of a perfused

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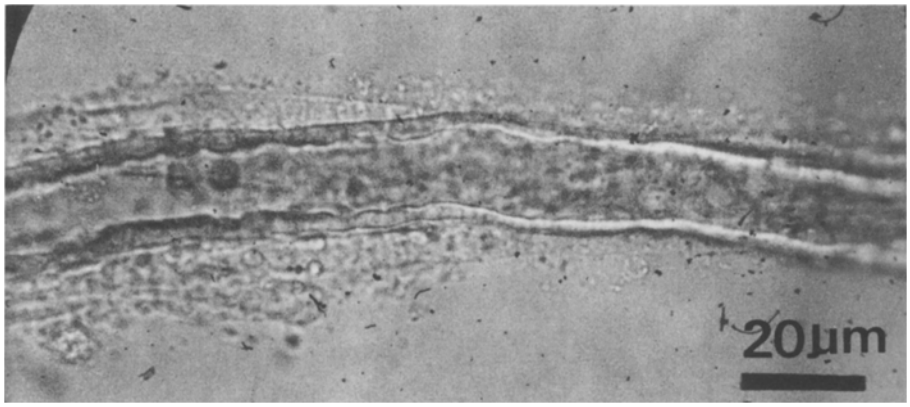

Fig. 1
A light microscope picture of a perfused papillary collecting duct isolated from a rat kidney

PCD is shown in Fig. 1. In order to prevent water evaporation from the bath, we constructed a bathing system which allows the bathing medium of flow continuously. We measured both diffusional water permeability (P_{dw}) and urea permeability (P_{urea}) simultaneously in the same preparation by adding ^{3}H-water (Amersham) and ^{14}C-urea (Amersham, Buckinghamshire, England) to the perfusate. Permeability coefficients (P) were calculated according to the following equation:

$$P = (\dot{V}_o/L)\ln(C_i^*/C_o^*) \quad (1)$$

where $\dot{V}_o$ is perfusion rate in ml s^{-1}, L is tubular length in cm, and C_i^* and C_o^* are the concentration of isotopes in perfusate and collected fluid, respectively. For comparison with other data, permeability coefficients were also expressed for unit of surface area by replacing L in Eq. (1) by S, surface area in cm^2.

We started the experiments after an equilibration period of about 60 min. The isolated renal tubules were perfused at rates ranging 30–50 nl/min. Tubular effluents were collected in constant-volume pipettes of 50–100 nl. Thus, the collecting periods for flux measurement ranged between 2–3 min. We used the following four protocols.

Protocol 1. We examined the effects of 0.1 mol/l glutaraldehyde treatment on vasopressin-stimulated P_{dw} and P_{urea} in eight tubules. After three samples had been collected in the control period, 10^{-9} mol/l arginine vasopressin (AVP) was added to the bath. An equilibration period for 5–10 min was followed by collection of three samples for the AVP period. Then the tubules were fixed for 5 min by adding 0.1 mol/l glutaraldehyde to the perfusate. Following elimination of AVP from the bath and of glutaraldehyde from the perfusate, three samples were collected in the absence of AVP during the post-glutaraldehyde period.

Protocol 2. We observed the effects of 0.1 mol/l glutaraldehyde treatment of the basal P_{dw} and P_{urea} in five renal tubules. After three samples were collected in the control period, tubules were fixed with 0.1 mol/l glutaraldehyde as in protocol 1 and three samples were collected during the post-glutaraldehyde period.

Protocol 3. We examined in four PCDs stimulated with AVP and fixed with glutaraldehyde whether P_{dw} and P_{urea} could be modified by 2.5×10^{-4} mol/l phloretin. After AVP-stimulated PCD segments were fixed with glutaraldehyde as above, we observed the effects of phloretin by adding the drug either to the lumen or to the bath. This protocol consisted of the following five periods: the control, phloretin in the lumen, recovery, phloretin in the bath, and the recovery period. In each period, three samples were collected. Between two successive periods, 5–10 min equilibration periods were interposed.

Protocol 4. We conducted five experiments in which we observed the effects of 2.5×10^{-4} mol/l phloretin on baseline P_{dw} and P_{urea} in PCD fixed with 0.1 mol/l glutaraldehyde. This protocol also consisted of five periods similar to protocol 3.

The mean of data from three samples was taken as the result for a given experimental condition. Then we calculated means ± SEM for each experimental period. Student's t-test for paired data was performed to evaluate the statistical significance of differences of means. Differences with a probability level of $P < 0.05$ were considered statistically significant.

Results

Effects of glutaraldehyde treatment on AVP-stimulated P_{dw} and P_{urea}

Protocol 1. We examined whether the glutaraldehyde treatment can preserve the vasopressin-stimulated P_{dw} and P_{urea} in the rat papillary collecting tubule. The results of eight experiments are summarized in Table 1. Individual data are shown in Fig. 2. Addition of 10^{-9} mol/l AVP to the bath increased P_{dw} and P_{urea} from 101.9 to 283.3×10^{-7} cm^2 s^{-1} ($P < 0.01$) and from 30.3 to 83.5×10^{-7} cm^2 s^{-1} ($P < 0.01$), respectively. When 0.1 mol/l glutaraldehyde was added to the perfusate in the presence of AVP in the bath, P_{dw} and P_{urea} were reduced slightly to 265.0 and 74.5×10^{-7} cm^2 s^{-1}, respectively. These values were 93.5% and 89.2% of the pretreatment values, suggesting that large proportions of the AVP-stimulated P_{dw} and P_{urea} were well preserved by glutaraldehyde fixation.

Effects of glutaraldehyde treatment on basal P_{dw} and P_{urea}

Protocol 2. We examined whether glutaraldehyde affects the basal levels of P_{dw} and P_{urea}. The results of five experiments

Table 1. Effects of glutaraldehyde on basal and vasopressin-stimulated diffusional water permeability (P_{dw}) and urea permeability (P_{urea})

	P_{dw}		P_{urea}	
	10^{-7} cm^2 s^{-1}	(10^{-5} cm s^{-1})	10^{-7} cm^2 s^{-1}	(10^{-5} cm s^{-1})
Group 1 (n = 8)				
Control	101.9 ± 10.76	(98.3 ± 15.24)	30.3 ± 2.24	(27.6 ± 2.67)
AVP 10^{-9} mol/l	283.3 ± 16.67*	(263.8 ± 22.67)*	83.5 ± 7.80*	(73.1 ± 6.50)*
0.1 mol/l glutaraldehyde	265.0 ± 14.47*,**	(247.2 ± 21.09)*,**	74.5 ± 7.15*,**	(64.9 ± 5.93)*,**
Group 2 (n = 5)				
Control	89.3 ± 6.18	(85.8 ± 7.60)	23.6 ± 4.49	(22.2 ± 3.96)
0.1 mol/l glutaraldehyde	96.1 ± 14.18	(93.0 ± 16.11)	20.5 ± 5.15	(17.4 ± 3.00)

The values in parentheses are expressed for surface area
* $P < 0.01$ as compared to control value
** $P < 0.01$ as compared to the values of the preceding periods

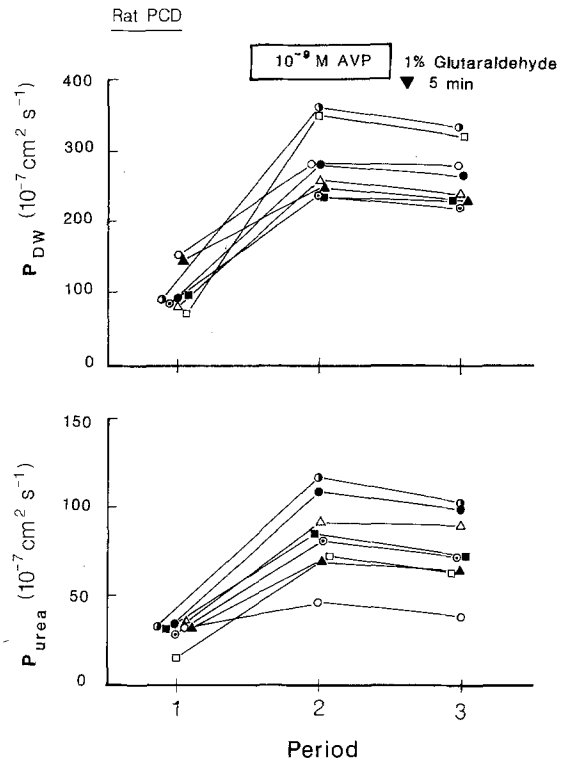

Fig. 2. Effects of glutaraldehyde fixation on vasopressin-stimulated diffusional water permeability (P_{dw}) and urea permeability (P_{urea}) of rat papillary collecting ducts. *AVP*, arginine vasopressin. 1% glutaraldehyde is equivalent to 0.1 mol/l

are summarized in Table 1. It is clear that addition of 0.1 mol/l glutaraldehyde to the perfusate did not affect the basal levels of these parameters.

Effects of phloretin on AVP stimulated and glutaraldehyde-fixed P_{dw} and P_{urea}

Protocol 3. We examined whether phloretin selectively inhibits P_{urea} in the AVP-stimulated and glutaraldehyde fixed PCD. The results are summarized in Table 2 and Fig. 3. Initially AVP-stimulated PCDs were pre-fixed with 0.1 mol/l glutaraldehyde for 5 min. Before the control period, both AVP and glutaraldehyde were removed from the bath and from the perfusate, respectively. Phloretin did not affect the vasopressin-stimulated P_{dw} whether it was added to the lumen or to the bath. By contrast, phloretin significantly reduced P_{urea} from 79.0 to 29.7×10^{-7} cm^2 s^{-1} and from 73.2 to 38.7×10^{-7} cm^2 s^{-1} when it was added to the lumen or to the bath, respectively. The effect of phloretin was reversible.

Effects of phloretin on glutaraldehyde fixed basal P_{dw} and P_{urea}

Protocol 4. As summarized in Table 2, phloretin at 2.5×10^{-4} mol/l did not affect P_{dw} whether it was added to the lumen or to the bath. By contrast, phloretin reversibly inhibited basal levels of P_{urea}, from 21.1 to 12.9×10^{-7} cm^2 s^{-1} when it was added to the lumen, and from 22.1 to 16.9×10^{-7} cm^2 s^{-1} when added to the bath. In this particular series of experiments, P_{dw} tended to increase as a function of time. In order to examine whether phloretin affected P_{dw} or not, we compared the values in phloretin period with the mean values of pre- and postphloretin periods. Changes in P_{dw} when phloretin was added to the lumen or to the bath were $-4.8 \pm 2.2 \times 10^{-7}$ cm^2 s^{-1} or $-0.2 \pm 2.4 \times 10^{-7}$cm^2 s^{-1}, respectively. Neither values were significantly different from zero. Thus, we conclude that phloretin does not change P_{dw}. We do not know why P_{dw} increased as a function of time. However, this was not due to the deterioration of epithelial integrity, for the simultaneously determined P_{urea} completely recovered to a reasonable control level when phloretin was eliminated.

Discussion

The urea permeability of the rabbit cortical collecting duct is quite low (0.3×10^{-5} cm s^{-1}) and not enhanced by vasopressin [23–25]. Rocha and Kokko [23] reported that P_{urea} of the rabbit PCD isolated and perfused in vitro was slightly higher (2.0×10^{-5} cm s^{-1}) but unaffected by vasopressin. On the other hand, using the technique of in vitro perfusion of PCD in rat kidney slices. Morgan and Berliner [18] reported that urea permeability was increased from 20 to 30×10^{-5} cm s^{-1}. More recently, Rocha and Kudo [24] found that in isolated perfused rat PCD vasopressin increased P_{urea} from 14 to 36×10^{-5} cm s^{-1}.

Table 2. Effects of phloretin on glutaraldehyde-fixed basal and AVP-stimulated diffusional water permeability (P_{dw}) and urea permeability (P_{urea})

	P_{dw}		P_{urea}	
	10^{-7} cm^2 s^{-1}	(10^{-5} cm s^{-1})	10^{-7} cm^2 s^{-1}	(10^{-5} cm s^{-1}
Group 3 ($n = 4$)				
AVP 10^{-9} mol/l				
0.1 mol/l glutaraldehyde	277.3 ± 27.84	(252.7 ± 25.29)	79.0 ± 7.96	(72.1 ± 7.59)
Phloretin (lumen) 2.5×10^{-4} mol/l	286.2 ± 29.58*	(260.8 ± 26.97)*	29.7 ± 3.66**	(27.2 ± 3.72)**
Recovery (1)	288.9 ± 25.56	(263.5 ± 23.89)	73.2 ± 7.05**	(66.8 ± 6.69)**
Phloretin (bath) 2.5×10^{-4} mol/l	289.3 ± 28.53	(264.1 ± 26.85)	38.7 ± 3.57**	(35.5 ± 3.85)**
Recovery (2)	304.2 ± 29.95	(277.2 ± 27.24)	74.7 ± 7.20**	(68.1 ± 6.75)**
Group 4 ($n = 5$)				
0.1 mol/l glutaraldehyde	96.1 ± 14.18	(93.0 ± 16.11)	21.1 ± 2.96	(20.1 ± 2.72)
Phloretin (lumen) 2.5×10^{-4} mol/l	107.5 ± 17.69*	(103.9 ± 19.78)*	12.9 ± 3.32*	(12.2 ± 3.01)*
Recovery (1)	128.1 ± 17.80**	(123.8 ± 20.10)**	22.1 ± 4.13*	(21.1 ± 3.91)*
Phloretin (bath) 2.5×10^{-4} mol/l	149.8 ± 17.31**	(144.5 ± 20.15)**	16.9 ± 3.42	(15.9 ± 2.93)
Recovery (2)	163.9 ± 15.85	(153.7 ± 19.53)	23.4 ± 4.01*	(22.3 ± 3.75)*

The values in parentheses are expressed for surface area.
* $P < 0.05$, ** $P < 0.01$ as compared to the values of the preceding periods

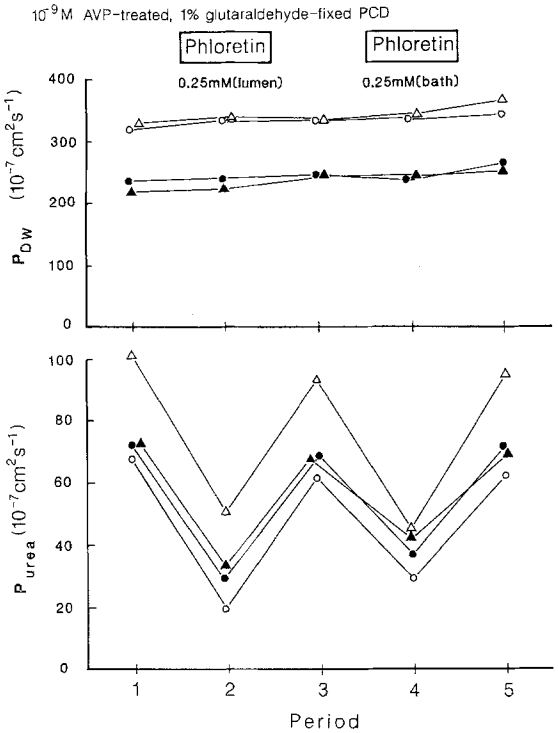

Fig. 3. Effects of phloretin on vasopressin-stimulated difussional water permeability (P_{dw}) and urea permeability (P_{urea}) in rat papillary collecting ducts

Thus, it seems that there are species difference and intranephron heterogeneity in the responsiveness of P_{urea} to the stimulation with vasopressin.

Therefore, it was necessary to choose the rat PCD in order to examine characteristics of the vasopressin-stimulated urea transport in mammals. In the present study, we confirmed the observations by Morgan and Berliner [18] and Rocha and Kudo [24] that vasopressin increases P_{dw} and P_{urea} in the rat PCD. Although the basal level of P_{urea} in present study was slightly higher than that reported by Rocha and Kudo [24] (28 vs. 14×10^{-5} cm s^{-1}), the response to vasopressin was much greater (73 vs. 36×10^{-5} cm s^{-1}). The same was true for P_{dw}: in the present study P_{dw} was increased from 102 to 283 $\times 10^{-5}$ cm s^{-1} by vasopressin, whereas in their study it was increased from 70 to 113×10^{-5} cm s^{-1}. We do not know at present, why our basal values for P_{urea} and P_{dw} were slightly higher than previously reported values. Although most of our studies started about 1 h after initiating the tubular perfusion, further prolongation of equilibration time did not affect the basal levels of these parameters.

The present study also demonstrated that both the vasopressin-stimulated P_{dw} and P_{urea} of mammalian papillary collecting ducts were fixed by the glutaraldehyde treatment as well. Although the glutaraldehyde treatment significantly reduced both parameters, the percent decreases were very small (6.5% for P_{dw} and 10.8% for P_{urea}). A similar tendency has also been observed for the amphibian urinary bladders [9, 20]. Thus, we conclude that the major components of the vasopressin-stimulated P_{dw} and P_{urea} were well preserved.

The increase in water permeability by vasopressin is a consequence of a series of biochemical events following to the stimulation of the adenylate cyclase and cyclic AMP system. The final step leading to an increase in water permeability across the luminal membrane is the insertion of as yet unidentified water channels into the membrane [4, 19, 22, 27]. The numbers of channels in the membrane may reflect a state of dynamic equilibration where both insertion and extrusion processes are taking place simultaneously [19, 27]. Such processes may be dependent on some cellular metabolic processes in which case it would be unreliable to characterize the water channels by observing the effect of changing temperature [10]. It has been reported that in the rabbit cortical collecting tubule, vasopressin-stimulated water permeability was decreased at 37° C but not a 25° C, although the reason for this was not estabilished [11]. The finding that glutaraldehyde fixes the vasopressin-stimulated P_{dw} and P_{urea} in the collecting tubule should be useful

for characterizing the transport units without problems of interference by some metabolically-dependent processes.

Another issue which we wished to address in this study was whether or not the function of glutaraldehyde-fixed transport can be modulated. In other words, does glutaraldehyde preserve vasopressin-induced transport channels in a flexible state? To answer this question, we chose phloretin which has been shown to inhibit urea transport in various biological membranes including red blood cells [17], amphibian urinary bladders [16], toad gall bladders [5] and rabbit proximal straight tubules [15]. The results clearly indicate that glutaraldehyde treated and AVP-stimulated P_{urea} is strongly inhibited with phloretin whether it is added to the bath or to the perfusate and that the inhibitory effects are reversible. Phloretin also inhibited the basal level of P_{urea} in glutaraldehyde-fixed PCD. This observation suggests that the urea transporters are fixed in a state which retains their responsiveness to inhibitors. Petrucelli and Eggena [21] reported that in toad urinary bladders, glutaraldehyde-fixed and vasopressin-stimulated P_{urea} was inhibited by urea and thiourea analogues. On the other hand, Hardy [12] reported that the glutaraldehyde treatment abolished the inhibitory effect of amiloride on P_{urea} in toad urinary bladders. Thus, the responsiveness of transporters after preservation by glutaraldehyde may differ depending on agents. This may be accounted for by differences in the mechanism of inhibition of each agent.

We observed that phloretin did not affect P_{dw}. This strongly suggests that the vasopressin-stimulated water channels are distinct from the urea channels. This fact has been well established in anuran membranes [2, 3, 26]. Therefore, the present observation suggests that papillary collecting duct are similar to anuran membranes in this respect.

In summary, we found that in the rat papillary collecting duct glutaraldehyde preserved vasopressin-stimulated P_{dw} and P_{urea} and that the glutaraldehyde-fixed P_{urea} was inhibited by phloretin in a reversible manner. Thus the glutaraldehyde fixation method may also provide a useful tool for studying vasopressin-stimulated transport processes under stable condition as in mammalian collecting duct.

Acknowledgements. We would like to express our thanks to Junichi Taniguchi, Ph.D. for his critical reading of this manuscript. The expert secretarial work of Miss Mari Wada is appreciated for preparation of this manuscript. This work was supported in part by a Research Grant for Cardiovascular Diseases (59-C8) from the Ministry of Health and Welfare of Japan.

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Received April 14/Accepted November 19, 1986