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Transport of Phosphate during Development by Renal Brush Border Membrane

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=Abstract=To elucidate the mechanism for higher tubular reabsorption of Pi (inorganic phosphate) observed in the young growing animals, vesicle preparations of proximal tubule brush border obtained from male Wistar rats of either 4-5 weeks (young) or 9-10 weeks (more mature) of age were used to assess the kinetic characteristics of the Na-Pi cotransporter.

No significant difference was noted in the affinity (Km) of this transport system, but the capacity (V_{max}) was about 2 times higher in the young than the more mature rat brush border membrane vesicles (BBMV).

The results demonstrate that renal adaptation in Pi transport in the proximal tubule brush border membrane during early developmental period is mainly due to higher capacity of the Na-Pi cotransport carrier.

However, physical forces along the proximal tubule brush border membrane and extrinsic factors such as growth hormone and parathyroid hormone may have additional role in the maintenance of positive Pi balance during the early developmental period *in vivo.*

Key words: ***Renal brush border membrane vesicles, Development, Na-Pi transport kinetics***

INTRODUCTION

It is well documented that plasma concentrations of Pi bear a direct relationship to the rate of growth both in human (Richmond *et al.* 1951; McCrory *et al.* 1952; Connelly *et al.* 1962) and in various animals (Altman and Ditter 1974). While it might be considered an advantage for growing cells to be exposed to a rich Pi environment, the mechanisms by which the high plasma levels are maintained in the developing subject are not well understood.

It is likely that the kidney contributes to the phenomenon and there is evidence to suggest that the renal response to various factors that regulate Pi handling vary with the stage of maturation (Connelly *et al.* 1962; Linarelli *et al.* 1972; David and Anast 1974). By clearance study, McCrory *et al.* (1952) suggested that low glomerular filtration rate (GFR) prevailing in young growing period in human, contributes substantially to the retention of phosphate.

However, studies done by Russo and Nash (1980) in beagles given various diets revealed that the rate of Pi reabsorption was higher in the young than in the adult animals and that both the plasma Pi concentration and the Pi excretion were independent of GFR. Furthermore, Caverzasio *et al.* (1982a) reported that maximal tubular reabsorption of phosphate (TRPi/ml GFR) was found to be significantly higher in the young growing (2 mo-old) than the adult (8-9 mo-old) male Wistar rats. This difference persisted even after thyroparathyroidectomy and was not associated with differences in GFR.

There is ample evidence that Pi is absorbed almost exclusively in the proximal tubule, especially the earliest portion (S_1 segment), at least in intact animals (Knox *et al.* 1977). In the proximal tubule of the kidney, it has become increasingly apparent that a link exists between Na and Pi transport, most evidently by the brush border membrane vesicle experiment (Hoffman *et al.* 1976).

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And the hypothesis has been put forward that the Na-gradient dependent Pi transport across the brush border membrane of the proximal tubule is the main limiting step in the regulation of the tubular transport of Pi based on the existence of an impressive correlation between the value of TRPi/ml GFR determined in the whole kidney and that of Na- Pi contransport (Stoll *et al.* 1979; Stoll *et al.* 1980).

In order to see whether there is a developmental change in Na-coupled Pi transport in the proximal tubule between the young and the adult kidneys, the experiment using brush border membrane vesicles from rat kidneys was carried out. Microvesicle preparation of the brush border membrane of the kidney seems the most suitable experimental material because of it's absence of metabolism and the ease with which the composition of the solutions on both sides of the membrane can be controlled (Kinne and Schwartz, 1978).

MATERIALS AND METHODS

Young (4-5 weeks old, body weight 75-110 gm) or more mature (9-10 weeks old, body weight 250-300 gm) male Wistar rats were used for the experiment. The rats were allowed free access to food (> 0.4% Pi diet) and water until the night before the experiment and to water until sacrifice of the animals. To get the chemical measurements for the whole animals, blood by intracardiac puncture and bladder urine were obtained right after the sacrifice by cervical dislocation.

Preparation of brush border membrane vesicles (BBMV)

BBMV were prepared by the calcium precipitation method described by Evers *et al.* (1978). Animals were sacrificed by a sudden cervical dislocation, following which the kidneys were removed rapidly and placed in 10cc of ice cold mannitol buffer (10 mM mannitol, 2 mM Tris at pH 7.1) for each gm of tissue. All succeeding steps were carried out at 0-4°C. For each experiment, 4-8 rats of each age were required in order to obtain a sufficient amount (≃3 gm) of superficial cortex. After the kidneys were removed, the superficial cortex (approximately 1/3 of the cortex width) was carefully isolated by hand cutting by a #10 blade and the tissue was homogenized (initial homogenate) for 3 minutes in a Sorvall Omni mixer.

Calcium chloride was added to the initial homogenate to give a final concentration of 10 mM, and the suspension was allowed to stand with occasional mixing for 15 minutes. Then a series of alternating low and high speed centrifugations were carried out to obtain the final brush border vesicle pellet. A sufficient volume of HEPES-mannitol buffer (100 mM Mannitol, 20 mM HEPES at pH 7.4) was added to give a protein concentration in the final suspension of 5-15 mg/ml. The mixture was aspirated 10 times through a 27 gauge needle and used for measurements of protein concentration, enzyme activities and for transport studies. Solutions were filtered through 0.45 μm filters and the containers were sterilized before use.

Analytical method

The purity of the BBMV preparations was determined by measuring alkaline phosphatase (Andersch and Szcaypinski 1947) and Na-K ATPase (Katz and Epstein 1967) content in both initial homogenates and final BBMV preparations. Protein concentrations were measured by Lowry method (1951) using bovine serum albumin as the standard. Inorganic phosphorus was measured by a modification of the Fiske and Subbarow (1925) method.

Calcium and creatinine were measured by Ca-atomic absorption spectrophotometer (Instrumentation Laboratory aa/ae spectrophotometer 257) and by Jaffe method respectively.

Transport studies

The incubation medium(mannitol 100 mM, NaCl or KCl 100 mM , HEPES 20 mM, at pH 7.4) with various concentration of Pi was used for the study. A sufficient quantity of $^{32}PO_4$-K_2HPO_4 was added to achieve concentrations of 0.1 to 3 mM Pi. Transport experiments were carried out essentially according to the method of Hopfer *et al.* (1973) at room temperature.

For the kinetics of the transport study, 10 sec. uptakes were carried out. The 10 sec. period was chosen because it was shown that the uptake of Pi was linear up to 15 sec (Lelievre-Pegorier *et al.* 1983) and also more reproducible at 10 sec. than at shorter times.

To see the overshoot uptake of Pi with inward Na gradient, the uptakes were terminated at 10 sec, 1 min, 4 min and 120 min by withdrawing 25 μl of the incubation mixture and adding it to 1cc of the ice cold stop solution (100 mM mannitol, 100 mM NaCl, 20 mM HEPES, 10 mM Arsenate at pH 7.4). The resultant suspension was rapidly passed through a 0.45 μm filter (Whatman) under continuous suction and washed with 3 ml of ice cold stop solution. The filters were dried out, covered

with 10 ml of Instagel (Packard Instrument Co, Inc.) scintillation fluid and counted in a liquid scintillation counter (Packard Tri-carb). All incubations were performed in triplicate. A sample of the incubation medium was also counted with each experiment to determine the specific activity. Chemical reagents of high purity were obtained from Sigma.

RESULTS

Whole animal data (Table 1)

Serum Pi level of the young rats (4-5 weeks old) was 4.81 ± 0.19 mg/dl (mean ± SEM), which was significantly ($P < 0.005$) higher than that of the more mature rats (9-10 weeks old). TRPi were 99.06 ± 0.52% and 90.2 ± 1.96% in the young and more mature rats respectively, which also was significantly higher in the young rats. On the other hand, serum creatinine and serum calcium levels were not different between the groups.

The data suggest that the animals used in this experiement were adequate for the purpose of the study.

Characteristics of membrane preparations (Table 2)

Protein concentrations of the young and more mature rat BBMV were 11.42 ± 1.1 mg/ml and 10.0 ± 0.76 mg/ml respectively, which were not different statistically. Alkaline phosphatase, brush border membrane enzyme, enrichment was 8.19 ± 0.19 in the young and 8.97 ± 0.54 in the more mature rat kidney BBMV, which were not statistically different and reasonably adequate for the study. Na-K ATPase, basolateral membrane enzyme, enrichment was also similar in both ages, and the contamination of basolateral membrane to the BBMV preparations for both ages was acceptable to perform the study.

Uptake study according to the incubation time

The uptake studies at the Pi concentration of 0.3 mM in the incubation medium according to the incubation time showed an overshoot and declined to the equilibrium value at 120 min. At 10 sec, the uptake with inward 100 mM Na gradient of BBMV from the young rat was 840 ± 67.2 (mean ± SEM) pmol/mg protein, which was significantly ($P < 0.005$) higher than that of the more mature rats BBMV (533 ± 50.4 pmol/mg protein). Both preparations showed peak uptakes at 1 min, which were 2020.2 ± 130.2 and 1197 ± 67.2 pmol/mg protein in the young and more mature rat BBMV re-

Table 1. Whole Animal Data

Age	4-5 weeks (n=10)	9-10 weeks (n=8)	P
Serum Pi(mg/dl)	4.81 ± 0.19*	3.57 ± 0.19	<0.005
Serum Cr(mg/dl)	0.35 ± 0.03	0.43 ± 0.04	n.s**
Serum Ca(mg/dl)	9.55 ± 0.25	9.16 ± 0.24	n.s.
Urine Pi/Cr	0.02 ± 0.002	0.81 ± 0.17	<0.005
TRPi (%)	99.06 ± 0.52	90.20 ± 1.97	<0.005

*mean ± SEM
**not significant

Table 2. Characteristics of Brush Border Membrane Vesicle Preparations

	4-5 weeks (n=15)	9-10 weeks (n=12)	P
Protein conc. (mg/ml)	11.42 ± 0.01*	10.00 ± 0.76	n.s.**
Alkaline phosphatase enrichment	8.19 ± 0.34	8.97 ± 0.54	n.s.
Na-K ATPase enrichment	0.35 ± 0.07	0.43 ± 0.08	n.s.

*mean ± SEM
**not significant

spectively. The values were also significantly different between the groups. Pi transport in the presence of inward 100 mM KCl gradient, which is diffusional Pi transport, was not different between the groups. The Pi uptake curve according to the incubation time for BBMV from both young and more mature rat kidneys, expressed in percent of the equilibrium values (120 min) of Na gradient uptake of the young rat BBMV, is shown in Figure 1. The equilibrium uptakes in both groups were not statistically different, which suggest that vesicular volumes were not different between the groups.

Kinetic studies

Fig. 2 is a double reciprocal plot (Lineweaver-Burke) of uptake studies at concentrations of ^{32}Pi in the media varying between 0.1 and 3 mM. Each point was plotted according to the uptakes at 100 mM NaCl gradient Pi uptakes subtracted 100 mM KCl gradient (diffusional) Pi uptake. In the young, the regression line ($y = 0.55x + 2.03$, $r^2 = 0.65$, $P < 0.05$) corresponds to Km = 0.27 mM and V_{max} = 4926 pmol/mg protein at 10 sec. In the more mature rats, the regression line ($y = 1.13x + 3.93$, $r^2 = 0.78$, $P < 0.05$) corresponds to 0.29 mM and V_{max} = 2544 pmol/mg protein at 10 sec. While the Km values were almost identical between the groups, V_{max} was almost 2 times higher in the young than that of the more mature rat preparations.

The result means that young rat kidney BBMV has a more efficient Na-Pi cotransport system.

DISCUSSION

Intrinsic to the process of growth is the maintenance of positive balance for a variety of substances including minerals like sodium, and phosphate *etc.* Pi is an important constituent of all body tissues, and is located mostly (85%) in the skeleton (Stoff 1982).

By the repeated observations that serum phos-

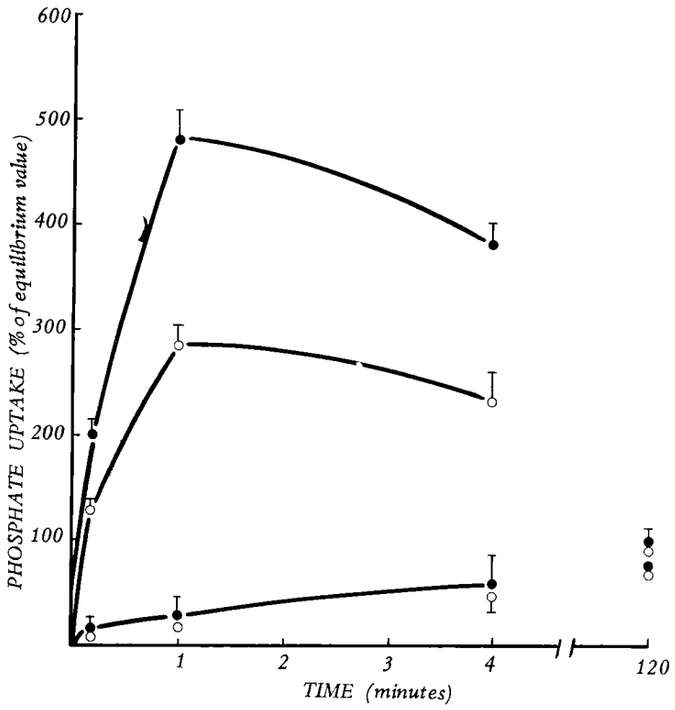

Fig. 1. The uptake ^{32}Pi by microvesicles of brush border membrane obtained from 4-5 weeks (●) and 9-10 weeks (○) old Wistar rat kidneys. The results are expressed in percent (mean ± SEM) of the equilibrium values of Na gradient uptakes of the young.
The upper two curves represent 0.3 mM ^{32}Pi uptake in the presence of 100 mM inwardly oriented NaCl gradient and the lower curve denotes ^{32}P uptake in the presence of 100 mM KCl in the incubation media.

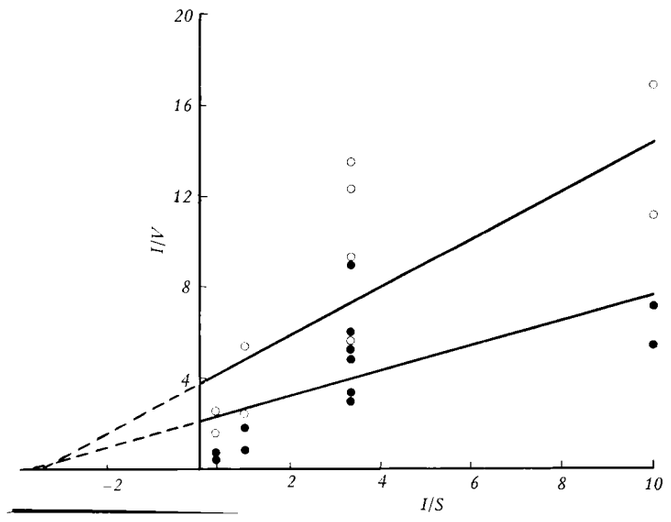

Fig. 2. Double reciprocal plots (Lineweaver-Burke) of the 100 mM Na gradient uptake studies at concentrations of ^{32}Pi in the media varying between 0.1 and 3 mM.
Each point represents the mean of triplicate uptake studies with a separate vesicle preparation.
In the young (lower curve, ●) the regression line ($y = 0.55x + 2.03$, $r^2 = 0.65$) corresponds to Km = 0.27 mM and V_{max} = 4926 pmol/mg protein at 10 sec, while in the adult (upper curve, ○) the regression line ($y = 1.13x + 3.93$, $r^2 = 0.78$) corresponds to Km = 0.29 mM and V_{max} = 2544 pmol/mg protein at 10 sec.

phate concentrations were higher in the growing subjects (Connelly *et al.* 1962; Brodehl *et al.* 1982), it's been suggested that an environment high in phosphate is essential to the accretion of new tissue (Spitzer *et al.* 1983).

Earlier investigators (McCrory *et al.* 1951; Brodehl *et al.* 1982), to define the underlying mechanism by which the kidneys of the growing subjects preserve the positive external balance of Pi, suggested that the retention of Pi is due to the lower rate of GFR in the growing subjects by clearance study. More recently, the role of tubular function in the maintenance of phosphate balance in the growing period has been more emphasized (Caversazio *et al.* 1982a; Johnson and Spitzer 1986).

In the kidney, filtered Pi is reabsorbed almost exclusively in the proximal tubule, at least in intact animals (Knox *et al.* 1977), mostly by the Na-Pi cotransport system, which is thought to be the main limiting step in the regulation of tubular transport of Pi (Baumann *et al.* 1975; Hoffman *et al.* 1976). Therefore, it is reasonable to speculate that higher Pi reabsorption in the tubule of the rapidly growing subject is expressed in the change of the Na-Pi cotransport system. Two mechanisms may be invoked to explain the more efficient tubular transport of Pi in the young than the more mature rats: a high affinity (lower Km) or a larger capacity (higher V_{max}) of the brush border membrane transport system for Pi.

Choi *et al.* (1982) could not find difference in the kinetics of the Na-Pi cotransport mechanism between newborn (<1 week old) and more mature (>6 weeks old) guinea pig kidney brush border microvesicle preparations even though TRPi (tubular reabsorption of Pi) was higher in the newborn guinea pigs. Instead, it was found that intracellular phosphate concentration of the newborn guinea pig kidneys measured by nuclear magnetic resonance technique was approximately half of that observed in more mature guinea pig kidneys. With the above findings, a conclusion was made that differences in driving force for Pi rather than in the kinetic characteristics of Na-Pi cotransporter account for the enhanced renal reabsorption of Pi observed in growing animals. To support the above observations, Caversazio *et al.* (1982a) reported that the maximal TRPi/ml GFR was markedly lower in adults (8-9 mo) than in young rats (2 mo) fed either a low (0.2%) or a normal (0.8%) diet. Studies with BBMV performed by these authours (1982b) in rats of similar ages indicate, however, that it is only under the conditions of a low phosphorus diet when the V_{max} is higher in the young than adult rats. Taken together, these experiments suggest that regulation of Pi reabsorption across the renal tubular epithelium involves factors other than kinetic properties of the Na-dependent Pi cotransport mechanism present in the luminal membrane or the proximal tubule.

However, the above two reports are against the general rule that changes in the renal handling of Pi induced by dietary maneuvers, removal of parathyroid gland, and chronic administration of 1, 25-OH-vitamin D_3 reveal a close correlation between initial rate of Na-dependent Pi uptake by the brush border membrane vesicles and renal reabsorption of Pi (Stoll *et al.* 1979; Kempson *et al.* 1980; Barret *et al.* 1980; Cheng *et al.* 1983).

To the contrary, our results in the male Wistar rats showed that capacity (V_{max}) of the Na-Pi cotransport system was about 2 fold higher in the BBMV of young rats than that of more mature rat kidney BBMV preparations, while the affinity (Km) of the system was essentially the same between the group. The difference in V_{max} is not due to differences in vesicle preparations because protein concentrations, enzyme enrichment didn't show significant difference between the two groups. The discrepancy of the result with the previous reports (Choi *et al.* 1982; Caverzasio *et al.* 1982b) cannot be fully explained. The following observations may be considered. Curiously enough, guinea pig newborns did not show higher serum Pi level than that of the adult (Choi *et al.* 1982), which may suggest that guinea pig is not an adequate experimental animal to study developmental Pi handling in the kidney. The animals of our experiment were fasted overnight, while the experiment of Caverzasio *et al.* (1982b) was done free access to food until the sacrifice. It may mean that our experiment was done under a low Pi diet, a situation in which, Caverzasio *et al.* (1982b) observed higher V_{max} in the young growing rats because the earliest detectable increase in Pi uptake by BBMV was reported to occur within 4 hour of dietary phosphorus deprivation (Levine *et al.* 1984). But in general, fasting reduces rather than increases V_{max} of Na-Pi cotransport in BBMV (Kempson *et al.* 1980).

An interpretation is made that the higher V_{max} we observed is more likely due to the higher density of the Na-Pi cotransport carrier in the young rat BBMV rather than differences in the intrinsic char-

acteristics of the transporter itself between the groups. Our speculation is that the density of the carrier may not increase to the degree of increase in proximal tubule brush border membrane surface area as Pi need decreases along with the growth of animals. Or the higher V_{max} in the young rat BBMV may be due to difference in the affinity of Na to the system (Amstutz *et al.* 1985). The phenomenon could also be due to differences in the rate of gluconeogenesis between the groups, which is known to modulate renal Pi reabsorption (Ou *et al.* 1981). Extrinsic factors known to modulate the renal handling of Pi even at the level of BBMV of the proximal tubule like parathyroid hormone (PTH) and growth hormone (GH) should also be considered in the interpretation of the above result. In neonatal rats (Gengler and Forte 1972) and guinea pigs (Johnson and Spitzer 1986), a degree of end organ unresponsiveness to PTH in terms of adenylate cyclase or phosphaturic response has been reported. Although no data on PTH is available for 4-5 week old Wistar rats, it is not likely that end organ unresponsiveness persists until these ages because chronic removal of the parathyroid gland in 2 mo-old Wistar rats was accompained by a marked increase in maximal tubular Pi reabsorption the same as would occur in adult rats (Caverzasio *et al.* 1982a).

A role of GH in the regulation of phosphate reabsorption was predicted on the basis of clinical observations made in patients with pituitary dwarfism and acromegaly (Corvilain and Abramov 1972). Moreover Na-stimulated Pi transport is enhanced in BBMV preparations from animals subjected to the chronic administration of GH (Hammerman *et al.* 1980). But acutely administered GH didn't elicit any changes in renal handling of Pi, suggesting that this hormone may act indirectly (Westby *et al.* 1977). It is possible to speculate that GH level may be higher in the young (4-5 weeks old) rats, and the observed higher V_{max} in the young rats may be, at least partly, due to higher GH level. Role of other factors such as insulin and vitamin D_3 cannot be discussed because of the lack of information.

Passive driving force may also influence the overall renal handling of Pi during development *in vivo*. The impact of downhill gradient on passive diffusion of Pi may be substantially greater in the newborn kidney tubule because of it's larger osmotic permeability (Larsson and Hoster 1972) and higher Pi concentration gradient between the tubular lumen and cell space (Choi *et al.* 1982).

In conclusion, the higher capacity of Na-Pi cotransport mechanism in the young (4-5 weeks old) seems to play the most significant role for the maintenance of higher serum phosphate level observed in these ages than more mature (9-10 weeks old) Wistar rats. Nevertheless other factors, for instance, GH, PTH and physical factors, may have an additional role at least *in vivo*.

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=국문초록=

신장의 Brush Border Membrane Vesicle (BBMV)을 통한 무기인 이동의 발육에 따른 변화

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최 용 · 고광욱

성장기에 있어서 혈청 무기인치는 성숙기에 비하여 현저히 높다는 것이 잘 알려져 있으며, 이는 성장기에 신세뇨관에서의 무기인 재흡수가 많기 때문이라고 믿어져왔다.

본 연구는 신장의 무기인 재흡수 기전에 가장 중요한 근위세뇨관 BBMV의 Na-Pi cotransport 기전이 성장기에 높은 무기인 혈청치를 유지하는데 어떤 역할을 하는지에 대하여 알아보고자 하였다. 생후 4-5주, 그리고 9-10주 된 Wistar rat 수컷들의 신장으로부터 Ca 침전법에 의하여 근위세뇨관 BBMV를 얻고, 이어 급속 여과술로 ^{32}Pi를 이용하여 무기인 이동을 관찰하고 kinetic analysis를 시행하였다.

4-5주된 쥐의 BBMV Na-Pi cotransporter의 Km은 0.27 mM, V_{max}는 4926 pmol/mg · 단백 · 10초 이었으며, 9-10주된 쥐의 BBMV에서는 Km 0.29 mM, V_{max} 2544 pmol/mg · 단백 · 10초였다. 즉 Km은 양자간에 차이가 없었으며 V_{max}는 4-5주된 쥐의 BBMV에서 약 2배 높았다.

위와 같은 결과로 급속 성장기의 높은 신세뇨관 무기인 재흡수에는 Na-Pi transporter의 높은 V_{max}가 가장 중요한 역할을 할 것으로 결론지었다. 그러나 실제 생체내에서는 Na-Pi cotransport 기전 이외에도 물리적인 요인 그리고 부갑상선 홀몬, 성장 홀몬 등이 성장기에 높은 혈청 무기인치를 나타내는데 어느정도 까지는 영향을 미칠 것으로 생각된다.