CHAPTER VI

ROLE OF L-ASCORBIC ACID IN RENAL CORTICAL LIPID PEROXIDATION, TGF-β1 AND GLUT 1 IN STZ-INDUCED DIABETIC RATS

Introduction

Under physiological conditions, glucose has been shown to undergo oxidation, producing protein reactive ketoaldehyde, hydrogenperoxide and highly reactive oxidant (Hunt and Wolff, 1991). It has been established *in vitro* studies that an increase in oxidative stress in glomerular mesangial cells is directly induced by high glucose concentration (Ha et al., 2000; Catherwood et al., 2002). In addition, enhanced lipid peroxidation in diabetic animals has been reported contributing to the development of diabetic nephropathy (Sharma et al., 1996; Riedle et al., 1997; Zhang et al., 1997; Ha et al., 1999; Clarkson et al., 2002). AA has been demonstrated as an antioxidant in preventing and inhibiting the renal damage induced by cytotoxic agents in rats (Appenroth et al., 1998; Greggi et al., 2000). Some studies showed that the supplementation of AA could increase the concentration of AA in the kidneys of diabetic rats (Siman et al., 1997; Lindsay et al., 1998).

Transforming growth factor-beta1 (TGF-β 1) is found as a key mediator of the development of diabetic kidney disease (Sharma et al., 1995). Neutralization of TGF-β by anti-TGF-β 1 antibody has been shown to attenuate kidney hypertrophy and the enhanced extracellular matrix gene expression in streptozotocin-induced diabetic mice (Sharma et al., 1996). Type II diabetic patients has been shown to increase in the renal production of TGF-β 1. The urinary levels of bioassable TGF-β1 were also significantly increased as compared with non-diabetic patients (Sharma et al., 1997). The long-term administration of neutralizing anti-TGF-β1 antibody can prevent glomerulosclerosis and renal insufficiency in diabetic *db/db* mice, the genetic model of non-insulin dependent diabetes mellitus (Ziyadeh et al., 2000). It was also noted that high glucose concentration stimulated TGF-β 1 expression in glomerular

mesangial cell and proximal tubular cell culture (Sharma et al., 1995). In addition, TGF-β 1-mRNA and protein overexpression, which are always found and lead to the increase in extracellular matrix synthesis in diabetic nephropathy, are stimulated by high glucose concentration both in vivo and in vitro studies (McLennan et al., 1994; Park et al., 2001). It has been elucidated that oxidative stress, which is caused by high glucose concentration, can induce diabetic nephropathy (Salahuddeen et al., 1997).

Glut 1 is found in most cell types, but primarily in red blood cells and the endothelial cells of blood vessels. Mesangial cells are also found Glut 1 which is important glucose transporter involving in the diabetic nephropathy (Rasch, 1979; Heilig et al., 1997; Inoki et al., 1999). It has been elucidated that not only Glut 1 but also TGF-β is upregulated by high glucose concentration in glomerular mesangial cells (Mogyorosi, et al., 1999). High glucose ambient increased Glut 1 expression and glucose transport activity when compared with physiologic glucose concentration. Treatment of rat mesangial cells with TGF-β can regulate Glut1 mRNA and protein levels and significant increase in glucose uptake. Cultured mesangial cells transducted with the human Glut 1 gene and thus over expressing the Glut 1 protein showed the marked increase in glucose uptake and the synthesis of extracellular matrix molecules. Both proteins of TGF-β and Glut 1 can influence the expression of one another (Koya et al., 1998).

However, the knowledge of the effect of AA on renal pathophysiology in diabetes mellitus *in vivo* study is not much available. Therefore, these experiments were performed in STZ-induced diabetic rats comparing with control rats to study whether AA, an antioxidant, affects the concentrations of malondialdehyde, a marker of the oxidative stress, and the concentrations of TGF-β1 and Glut1 in renal cortex in various progression of the diabetic nephropathy.

Materials and methods

The studies were performed in 96 male Sprague-Dawley rats weighing 180-220 gram. The experiments were divided into 4 groups of 4 observation periods as the experimental designs in Charpter III. At the end of the renal function experiments, each animal was subjected to collect the kidneys. The renal cortex homogenate was obtained by the method described in the issue 3.3 in Chapter III. Malondialdehyde concentrations of CON, CON-AA, STZ and STZ-AA of all observation times were determined by the method of thiobarbituric acid reaction (Ohkawa et al., 1979) following the method mentioned in the issue 3.3 in Chapter III.

To study the changing in the TGF-β1 concentration in renal cortex, TGF-β1 ELISA kit (DRG Instruments GmbH, Germany) was used to assay TGF-β1 protein based on the sandwich principle as followed the method in the issue 3.4 of Chapter III. The diluted homogenate (1:100) and the serial concentrations of TGF-β1 standard were added to the antibody coated microtiter wells. After the first incubation over night (16-18 hours), the unbound sample material was removed by washing with diluted wash solution. Then a monoclonal mouse antigen anti TGF-β1 antibody, a biotinilated anti mouse IgG antibody and streptavidin-HRP Enzyme complex were incubated in succession. An immuno enzyme sandwich complex was formed. The unbound conjugate was removed by washing. Subsequently TMB Substrate Solution was added. After a definite time color development was stopped by addition of stop solution and the absorbance at 450 nm was measured with a microtiterplate reader. The intensity of the color development was proportional to the TGF-β1 concentration in the sample.

The concentrations of MDA and the protein of TGF-β1 were corrected with milligram of total protein in the renal cortex homogenate.

Glut 1 protein in renal cortex homogenate was assayed by ELISA based on the indirect ELISA principle as followed the method in the issue 3.5 of Chapter III. The microtiter wells were coated with .primary antibody for Glut1 0.15 μg per well and incubated at 4°C overnight (16-18 hours). After the overnight incubation, the coated plate was washed with PBST washing solution and blocked with 1% gelatin and incubated at room temperture 2 hours. To confirm the change in Glut1 of the control and experimental groups, two amounts of total protein of the samples were fixed at 25

and 40 μg/well. Ater washing, 25 and 40 μg total protein of renal homogenate samples were added to the coated microtiter wells. After the incubation of 2 hours, the unbound sample material was removed by washing with PBST. Then, 50 μl of rabbit anti mouse IgG antibody-HRP conjugated was incubated in each well for 1 hour to form an immunoenzyme complex.. The unbound conjugate was removed by washing. Subsequently, OPD substrate solution was added. After a definite time color development was stopped by addition of stop solution and the absorbance at 492 nm was measured with a microtiterplate reader. The intensity of the color development was reverse proportional to the Glut 1 concentration in the sample. The histograms of OD and μg of total protein of control and experimental groups were examined.

Statistics

All values were expressed as means with standard deviations. Statistical comparisons among groups in the same observation periods were analyzed by ANOVA and using Least significant difference (LSD) or Thamhane's as the post hoct tests. The significant difference was indicated at p-value < 0.05.

Results

MDA concentration

The concentrations of malondialdehyde in renal cortex homogenate of CON, CON-AA, STZ and STZ-AA at the week 4, 8, 16 and 24 are presented in Table 6-1 and Figure 6-1.

At week 4 of the observation times, MDA of both diabetic groups were increased comparing with CON and CON-AA but no significant difference was seen (2.1 ± 1.21 and 2.2 ± 1.1 nmole/mg protein in STZ and STZ-AA *vs* 1.2 ± 0.6 and 1.6 ± 0.8 nmole/mg protein in CON and CON-AA, respectively). At week 8 of the observation times, MDA of both diabetic groups were increased (p < 0.05) as compared with CON (2.5 ± 0.4 and 2.2 ± 0.2 nmole/mg protein in STZ and STZ-AA *vs* 1.8 ± 0.5 nmole/mg protein in CON, respectively). In addition, CON-AA was seen the significant increase in MDA over CON (2.3 ± 0.2 *vs* 1.8 ± 0.5 nmole/mg protein). At week 16 of the experimental periods, MDA of STZ trended to increase as

compared with CON and CON-AA (2.7± 0.5 *vs* 2.3 ± 0.5 and 2.4 ± 0.3 nmole/mg protein, respectively) but it was not statistically significant. Interestingly, the diabetic rats with AA supplementation showed the decrease ($p < 0.05$) in MDA concentration in renal cortex after the supplementation of AA for 16 weeks of diabetic condition compring with diabetic rats without the treatment (1.92 ± 0.40 nmole/mg protein in STZ-AA *vs* 2.7 ± 0.5 nmole/mg protein in STZ). At week 24 of the observation times, both diabetic groups with or without AA supplementation were significantly increased in MDA concentration in the renal cortex as compared with both control groups at $p < 0.05$ (1.9 ± 0.1 and 1.8 ± 0.23 nmole/mg protein in STZ and STZ-AA *vs* 1.6 ± 0.2 and 1.6 ± 0.1 nmole/mg protein in CON and CON-AA, respectively). The effect of the supplemental AA on the decrease in lipid peroxidation was not seen at week 24.

TGF-β1 concentration

The concentrations of TGF-β1 in renal cortex homogenate of CON, CON-AA, STZ and STZ-AA at week 4, 8, 16 and 24 are presented in Table 6-2 and Figure 6-2.

At week 4 of the experimental periods, the concentration of TGF-β1 in the renal cortex were not statistically significant different among groups (351.3 ± 106.70 and 379.2 ± 113.6 pg/mg protein in CON and CON-AA *vs* 319.7 ± 88.1 and 340.0 ± 111.8 pg/mg protein in STZ and STZ-AA, respectively). At week 8 of the experimental periods, TGF-β1 in the renal cortex of STZ was markedly increased as compared with CON and CON-AA at $p < 0.05$ (601.5 ± 140.7 *vs* 411.4 ± 71.2 and 482.7 ± 60.1 pg/mg protein, respectively). In STZ-AA, the TGF-β1 concentration was increased as compared with CON at $p < 0.05$ (521.1 ± 70.9 *vs* 411.4 ± 71.2 pg/mg protein) but it was not significantly different as compared with CON-AA (482.7 ± 60.1 pg/mg protein). At week 16, only STZ showed a significant increase in the TGF-β1 concentration in the renal cortex as compared with those in CON and CON-AA (645.0 ± 128.7 *vs* 497.4 ± 85.8 and 489.6 ± 39.2 pg/mg protein). The diabetic rats with AA supplementation was significantly decreased in the renal cortical TGF-β1 concentration as compared with diabetic rats without supplemental AA at $p < 0.05$ (468.3 ± 85.4 *vs* 645.0 ± 128.7). It was not significantly different as compared with CON and CON-AA. At week 24, the result of STZ was the same as

those at the week 8 and week 16. The TGF-β1 concentration was significantly increased as compared with CON and CON-AA at $p < 0.05$ (473.4 ± 48.6 *vs* 395.3 ± 60.4 and 373.8 ± 50.6 pg/mg protein, respectively). STZ-AA had significant difference of TGF-β1 concentration as compared with CON-AA at $p < 0.05$ (455.1 ± 83.3 *vs* 373.8 ± 50.6 pg/mg protein) but it was not statistically different as compared with CON and STZ.

Glut 1 concentration

The absorbance histograms of CON, CON-AA, STZ and STZ-AA at week 16 are shown in Table 6-3 and Figure 6-3.

The absorbances of CON, CON-AA and STZ were not significantly different in both assays of 25 and 40 μg of total protein. The OD of STZ-AA was significantly decreased (or increase in concentration) ($p < 0.001$) as compared with those of CON, CON-AA and STZ in both assays. At 25 μg of total protein, OD of STZ-AA was 1.23 ± 0.10, significantly decreased ($p < 0.001$) as compared with 1.38 ± 0.03 of CON, 1.40 ± 0.04 of CON-AA and 1.39 ± 0.03 of STZ. At 40 μg of total protein, OD of STZ-AA was 0.87 ± 0.05, significantly decreased ($p < 0.001$) as compared with 1.08 ± 0.06 of CON, 1.02 ± 0.06 of CON-AA and 1.03 ± 0.07 of STZ.

Table 6-1 Malondialdehyde concentration in renal cortex of streptozotocin-induced diabetic rats and control rats with or without L-ascorbic acid supplementation at week 4, 8, 16 and 24 of the experimental period (n=6).

Groups	Malondialdehyde concentration (nmole/mg protein)			
	week 4	**week 8**	**week 16**	**week 24**
CON	1.2 ± 0.6	1.8 ± 0.5	2.3 ± 0.5	1.6 ± 0.2
CON-AA	1.6 ± 0.8	2.3 ± 0.2[a]	2.4 ± 0.3	1.6 ± 0.1
STZ	2.1 ± 1.2	2.5 ± 0.4[a]	2.7 ± 0.5	1.9 ± 0.1[ab]
STZ-AA	2.2 ± 1.1	2.3 ± 0.2[a]	1.9 ± 0.4[c]	1.8 ± 0.3[ab]

Mean ± SD

[a] compared with CON at the same column, $p < 0.05$

[b] compared with CON-AA at the same column, $p < 0.05$

[c] compared with STZ at the same column, $p < 0.05$

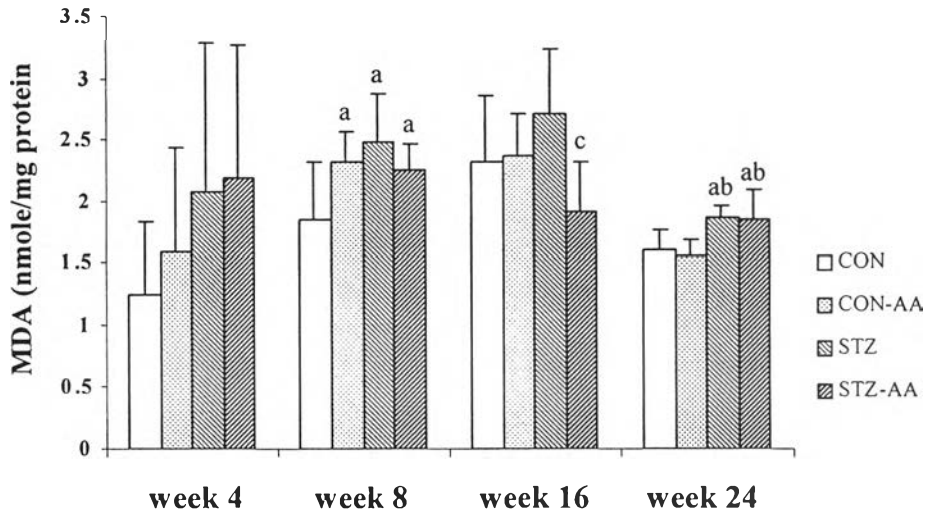

Figure 6-1 Alterations of MDA concentrations in renal cortex of streptozotocin-induced diabetic rats and control rats with or without L-ascorbic acid supplementation at week 4, 8, 16 and 24 of the experimental periods. All values are means ± SD. Statistically significant differences are indicated by [a] compared with CON at each period, $p < 0.05$; [b] compared with CON-AA at each period, $p < 0.05$ and [c] compared with STZ at each period, $p < 0.05$.

Table 6-2 Transforming growth factor-β 1 (TGF-β 1) concentration in renal cortex of streptozotocin-induced diabetic rats and control rats with or without L-ascorbic acid supplementation at week 4, 8, 16 and 24 of the experimental periods (n=6).

Groups	TGF-β 1 concentration (pg/mg protein)			
	week 4	**week 8**	**week 16**	**week 24**
CON	351.3 ± 106.7	411.4 ± 71.2	497.4 ± 85.8	395.3 ± 60.4
CON-AA	379.2 ± 113.6	482.7 ± 60.1	489.6 ± 39.2	373.8 ± 50.6
STZ	319.7 ± 88.1	601.5 ± 140.7[ab]	645.0 ± 128.7[ab]	473.4 ± 48.6[ab]
STZ-AA	340.0 ± 111.83	521.1 ± 70.9[a]	468.3 ± 85.4[c]	455.1 ± 83.3[b]

Mean ± SD

[a] compared with CON at the same column, $p < 0.05$

[b] compared with CON-AA at the same column, $p < 0.05$

[c] compared with STZ at the same column, $p < 0.05$

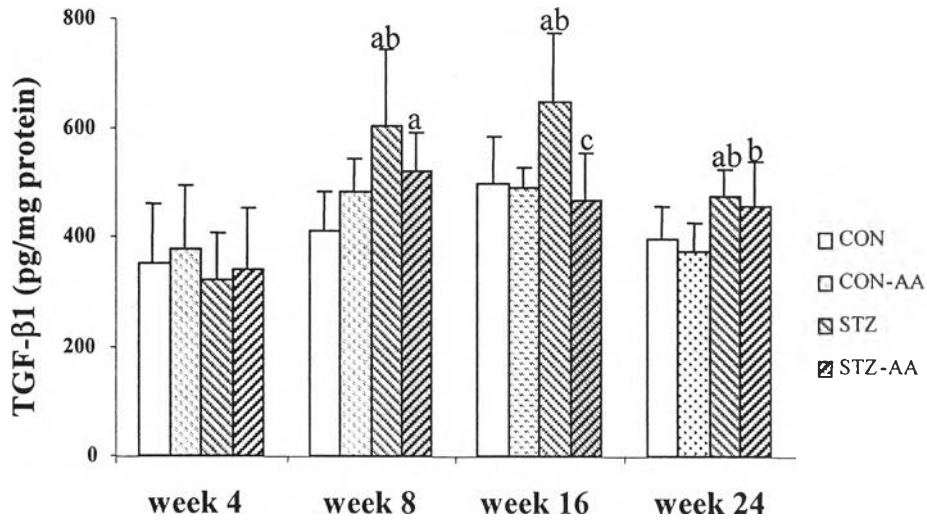

Figure 6-2 Alterations of renal cortical transforming growth factor -β1 concentrations of streptozotocin-induced diabetic rats and control rats with or without L-ascorbic acid supplementation at week 4, 8, 16 and 24 of the experimental periods. All values are means ± SD. Statistically significant differences are indicated by [a] compared with CON at each period, $p < 0.05$; [b] compared with CON-AA at each period, $p < 0.05$ and [c] compared with STZ at each period, $p < 0.05$.

Table 6-3 Absorbance at 492 nm of Glut 1 in 25 and 40 μg of total protein in renal cortex homogenate of streptozotocin-induced diabetic rats and control rats with or without L-ascorbic acid supplementation at week 16 of the experimental periods (n=6).

Groups	Absorbances of Glut1	
	OD per 25 μg of total protein	**OD per 40 μg of total protein**
CON	1.38 ± 0.03	1.08 ± 0.06
CON-AA	1.40 ± 0.04	1.02 ± 0.06
STZ	1.39 ± 0.03	1.03 ± 0.07
STZ-AA	1.23 ± 0.10 [a, b, c]	0.87 ± 0.05 [a, b, c]

Mean ± SD

[a] compared with CON at the same column, $p < 0.001$

[b] compared with CON-AA at the same column, $p < 0.001$

[c] compared with STZ at the same column, $p < 0.001$

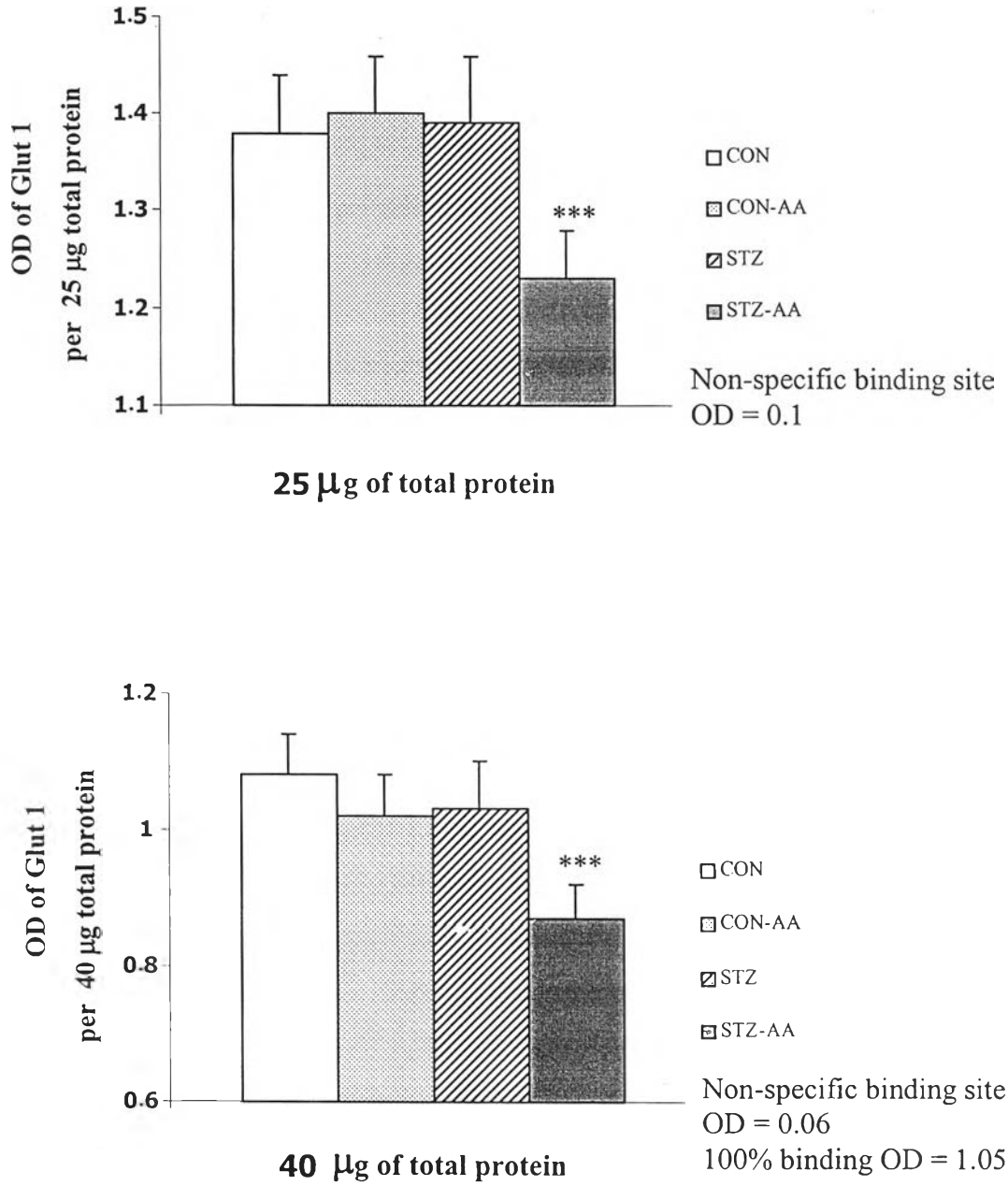

Figure 6-3 Absorbances of Glut 1 at 492 nm, comparisons of OD of the renal cortical Glut 1 concentrations of streptozotocin-induced diabetic rats and control rats with or without AA supplementation at week 16 at the levels of 25 and 40 µg of total protein. All values are means ± SD. Statistically significant differences are indicated by *** compared with CON, CON-AA and STZ, $p < 0.001$

Discussion

It has been reported that an increase in the blood glucose concentration results in the occurrence of systemic oxidative stress and causes renal abnormalities in diabetes mellitus (Hunt, 1991). The objective of this study was to study the effect of the supplemental AA being able to improve renal pathophysiology. To clarify the study, this experiment was performed to find out whether the mechanism of AA action is via the decrease in oxidative stress, which normally occurs in diabetes mellitus (Wohaieb et al., 1987), and the inhibition TGF-β 1 overexpression. The experiments were carried out in STZ-induced diabetic rat kidneys. MDA, a marker for lipid peroxidation, was determined a concentration in the renal cortex which is the site of glomerulosclerosis. In the present study, the renal cortical MDA concentrations in diabetic rats were increased as compared with the controls of all experimental periods. There were slightly increased in the MDA concentrations in diabetic rats at week 4 and noticeable at week 8 and week 24 (Table 6-1). This result agrees with the study of Asayama et al. (1989) who studied lipid peroxidation in short term of 2 weeks STZ-induced diabetic rats. Interestingly, the present result showed a decrease in the MDA concentration in diabetic rats after AA supplementation for 16 weeks. These results were coincided with the report of Kedziora-Kornatowska et al. (2003), which found a decrease in the MDA level in the kidney of diabetic rats after 6 and 12 weeks of experiments. However, this effect was not apparent in STZ-AA at week 24 in the present study. It might be due to a decrease in α-tocopherol (vitamin E) in diabetes mellitus. Vitamin E has been known to be a predominant antioxidant of lipid peroxidation accompanying with AA to eliminate reactive oxygen species in the inhibition of lipid peroxidation. The lack of this process may occur in diabetes mellitus at the severe stage of diabetes. It is probably the reason for the decrease in the renal cortical MDA concentration, which was not reveal in STZ-AA at week 24 (Je et al., 2001). However, MDA is only a marker of lipid peroxidation. Oxidative stress in diabetes is involved to an imbalance of antioxidants and other reactive oxygen species including oxygen free radicals, super nitrite, H_2O_2, $NO^{-\bullet}$, $OH^{-\bullet}$ etc. Those may be also affected by AA. Therefore, the present experiment indicates that AA is capable to decrease lipid peroxidation in the renal cortex of STZ-induced diabetic rats after AA supplementation for 16 weeks.

The TGF-β 1 production has been demonstrated to induce in the diabetic condition (Ziyadeh et al., 2000). In the present study, the TGF-β 1 overexpression in the diabetic rats was inhibited by the supplementation of AA. At week 4 after the diabetic induction, the renal cortical TGF-β 1 concentrations were not different among groups. The renal cortical TGF-β 1 concentrations were markedly increased in both diabetic groups over the control group at week 8 after the diabetic induction and prolong to the week 16 and 24 (Table 6-2). It indicates that four weeks after the diabetic induction is the early stage of diabetic stage, which was induced by streptozotocin injection. The result implied that the production of TGF-β 1 to develop the diabetic nephropathy might be in the stimulated process. Some evidences indicated that TGF-β 1 mRNA expression was increased in the first month of the diabetes (Makino, 2003; Ye and Li, 2004). Four weeks following, the levels of TGF-β 1 were markedly increased at week 8 to week 24 after the diabetic induction. This result agrees with an evidence of the glomerular expansion and thickening in the glomerular basement membrane which occurred after 8 weeks of the diabetic induction (Osterby, 1992; Liu et al., 2003). However, in the present study, the percentages of kidney weight to body weight have been significantly increased since week 4 of the diabetes (Table 4-2). These changes were probably due to the body weight loss in an improper proportion of the kidney weight. Since week 8 until week 24, the control rats were growing up while STZ groups had the less weight gained. In spite of the less body weight in STZ groups in comparison with the control groups, the kidney weights in STZ were slightly higher than those of the controls, resulting in an increase in the ratios of kidney weight to body weight. This result corresponds with the increase in the renal cortical TGF-β 1 concentration in STZ groups of the experimental periods (Table 6-2). The renal cortical TGF-β 1 concentrations of STZ groups were significantly increased as compared with those of CON groups from week 8 until week 24 after the diabetic induction. These results are in accordance with the results of the increase in the percentage of kidney weight respected to body weight. However, in the present study, no significant difference of the renal cortical TGF-β 1 concentration among groups at week 4 was observed while the percentages of kidney weight respected to body weight were significantly increased. Interestingly, at week 16 of diabetes with AA supplementation, the renal cortical TGF-β 1

concentration in STZ-AA was significantly declined as compared with that of STZ. According to the decrease in the percentage of kidney weight to body weight in STZ-AA. AA was able to decrease the nephropathic mediator, TGF-β 1, in STZ rats after the supplementation of AA for 16 weeks. This result agrees with the previous study with Western blot technique , which demonstrated for the increase in TGF-β of diabetic rats treated with vitamin C (McLennan et al., 1994; Craven et al., 1997; Park, 2001). However, at week 24 of AA supplementation, the increase in the renal cortical TGF-β 1 concentration of STZ-AA was not different from those of STZ and CON. This implies that the critical period for the beneficial effect of AA to decrease the TGF-β 1 concentration in the renal cortex in STZ-induced diabetic rats was within sixteen weeks of AA supplementation. Supplementation of AA longer than 16 weeks did not reveal the effectiveness of AA. The variation of the results may depend on the severity of diabetes mellitus. It is necessary to have other therapy simultaneously to retain the highest beneficial effect of AA supplementation (Futrakul et al., 2003). In addition, the result of the decrease in renal cortical TGF-β 1 concentration supports the result of the decrease in the renal cortical MDA concentration. This indicates that AA plays a role in the inhibition of a linkage of oxidative stress and TGF-β 1 production.

In the present study, it showed the increase in OD of the animals in STZ group, meaning the decrease in the concentration of Glut 1 in the renal cortex at week 16. The present result did not show the overexpression of Glut1 unlike as the previous studies in mesangial cell culture (Mogyorosi et al., 1999; Inoki et al., 1999; Heilig et al., 2001). However, it cannot conclude for no increase in the Glut 1 production in diabetic rats because the decrease in OD of STZ-AA was seen in the present study which indicates the increase in Glut 1 in diabetic rats even if AA was supplemented. To eliminate a false negative result possibly caused by too small amount of total protein, the samples were re-evaluated with more amount of total protein to confirm the present result. If the overexpression of Glut 1 is present, the apparent renal pathology in diabetic rats (see Chapter VII) is a possible explanation of the decreased Glut1 expression in the present study. The renal sclerosis results in the renal cell damage and dysfunction of Glut 1 synthesis. So the expression of Glut1 seems not to be different from the control rats or may be less (Table 6-3). On the

contrary, Glut 1 overexpression of STZ-AA was seen in this study. Two aspects should be considered, firstly, both STZ-AA and STZ were diabetes mellitus but the Glut 1 expressions were opposite. It implies that AA much possibly affects the renal cell to increase the Glut 1 production. The increase in Glut 1 protein indicates the amelioration of renal cell dysfunction in diabetic rats. A reasonable explanation for the increase in Glut 1 is to facilitate AA uptake to the cells. Since the diabetic cells are attacked by many free radicals, AA is more taken up to neutralized the free radical. This result indicates that the beneficial effect of AA on the diabetic renal cells are consistent with the amelioration of renal dysfunction (see Chapter IV) and renal pathology (see Chapter VII).

In conclusion, the present study indicates that AA is a capable antioxidant to suppress TGF-β1 production in diabetic rats. The mechanism of the effect of AA on TGF-β1 suppression is involved in the decrease in the oxidative stress and the increase in Glut 1 production for the enhancement of AA uptake.