# CHAPTER V

# ROLE OF L-ASCORBIC ACID IN RENAL MITOCHONDRIAL ACTIVITY IN STZ-INDUCED DIABETIC RATS

## Introduction

It has been reported that not only the glomerular part of the kidney develop sclerosis but also the renal tubular part (Wang et al., 2001). It has been reported that renal tubular functions in diabetes mellitus show the increase in absolute proximal tubular fluid reabsorption (Bark et al, 2001), but decrease in distal tubular reabsorption (Ward et al., 2001 and Slomowitz et al., 2002). Aldose reductase and polyol pathway as well as advanced glycation end products enhance the hyperglycemia-induced cellular impairment in renal tubular cells (Dunlop, 2000). High level of advanced glycation end products in hyperglycemic condition has direct toxicity for renal mitochondria. It has an inhibitory effect on both the tricarboxylic acid cycle and the electron respiratory chain (Rosca et al., 2002). In addition, the advanced glycation end products activating the proinflammatory gene products, such as interleukin-6 (IL-6), play a role in damaging the renal tubule (Morcos et al., 2002). Therefore, these experiments were performed *in vitro* to study renal mitochondrial activity in the early stage (8 weeks) and chronic stage (24 weeks) of diabetes mellitus. Furthermore, a study of the effect of supplemental AA on renal mitochondrial activity was carried out in control and in STZ-induced diabetic rats to find out whether supplemental AA improve renal mitochondrial activity.

## Materials and methods

The studies were performed on 32 male Sprague-Dawley rats weighing 180-220 gram. The experiments were divided into 4 groups of either week 8 or week 24 of the experimental periods. All animals were studied renal mitochondrial activity using the method of Malis and Bonventre (1986). The kidneys were immediately isolated, removed the adhering fat, cut into small pieces and homogenized in ice-cold homogenizing buffer to access the renal mitochondrial homogenate as the procedure

mentioned in the issue 3.2 of CHAPTER III. The mitochondrial activity was carried out in a close chamber with Clark-type electrode and continuously stirred by magnetic stirring bar at 25°C. The mitochondrial oxygen consumption ($Vo_2$) was measured using an oxygen consumption monitor (YSI Model 5300, Biological oxygen monitor. Scientific Division, Yellow Springs Instrument Co., Inc.). Respiration control index (RCI) and P/O ratio were determined. Moreover, the rates of mitochondrial respiration in Stage 3 and 4 were measured as described in the issue 3.2 of Chapter III

## 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) as the post hoct tests. The significant difference was indicated at $p$-value $< 0.05$.

## Results

The experiments were carried out to study renal mitochondrial activity including the rates of oxygen consumption in Stage 3 and Stage 4 of the mitochondrial respiration, the respiratory control index and P/O ratio at week 8 and 24 of the observation periods as shown in Table 5-1 to 5-2 and Figure 5-1 to 5-4

At week 8 of the observation periods, when glutamate and malate were used as the substrates in the mitochondrial respiration, the rates of oxygen consumption in Stage 4 was about 9 to 10 ng atom $O_2$/min/mg protein. No significant difference of the oxygen consumption rate in Stage 3 was seen among groups. The values of RCI in STZ ($6.3 \pm 1.7$) and STZ-AA ($6.3 \pm 1.3$) were not significantly different as compared with that of CON ($7.5 \pm 1.9$). These values were significantly ($p < 0.05$) low as compared with that of CON-AA ($9.5 \pm 2.7$). P/O ratios were about 2.0 with non-significant difference among groups at the early stage of diabetic condition as shown in Table 5-1.

When succinate was used as the substrate in the mitochondrial respiration, the results were similar as the case of glutamate and malate as the substrates. Namely, the rate of oxygen consumption at Stage 3 and 4 of the mitochondrial respiration in

both diabetic groups during Stage3 was not significantly different from both control groups as shown in Table 5-2. The value of RCI did not show a significant difference among groups. The values of RCI were about 3 to 5 in the case of succinate as the substrate. P/O ratios of all groups were about 1 to 2, which were not markedly different among groups.

At week 24 of the experimental periods, when glutamate and malate were used as substrates for the mitochondrial respiration, the rates of oxygen consumption at Stage 3 and 4 of the mitochondrial respiration were significantly increased in both diabetic groups as compared with both control groups. In Stage 4, oxygen consumption rates of STZ (10.4 $\pm$ 1.6 ng atom $O_2$/min/mg protein) and STZ-AA (11.3 $\pm$ 1.1 ng atom $O_2$/min/mg protein) were significantly ($p < 0.05$) increased as compared with those of CON (7.4 $\pm$ 0.9 ng atom $O_2$/min/mg protein) and CON-AA (7.5 $\pm$ 1.9 ng atom $O_2$/min/mg protein). In Stage 3, the rates of oxygen consumption in STZ (62.4 $\pm$ 12.8 ng atom $O_2$/min/mg protein) and STZ-AA (62.6 $\pm$ 8.3 ng atom $O_2$/min/mg protein) were significantly increased ($p < 0.05$) as compared with CON (43.3 $\pm$ 5.1 ng atom $O_2$/min/mg protein) and CON-AA (36.4 $\pm$ 10.7 ng atom $O_2$/min/mg protein). The values of RCI did not show any significant differences among groups. RCI of the experimental groups were about 5 to 6 in the case of glutamate and malate as the substrates. P/O ratios of STZ (2.2 $\pm$ 0.5) and STZ-AA (2.0 $\pm$ 0.3) were significantly ($p < 0.05$) increased as compared with CON-AA (0.9 $\pm$ 0.4). The control rats with AA supplementation for 24 weeks (CON-AA) had the significant ($p < 0.05$) decrease in P/O ratio (0.9 $\pm$ 0.4) as compared with that of CON (1.8 $\pm$ 0.5). No significant difference of those results was seen between STZ and STZ-AA.

In the case of succinate as the substrate in the mitochondrial respiration, the rate of oxygen consumption in Stage 4 was significantly ($p < 0.05$) increased in STZ-AA (28.1 $\pm$ 1.6 ng atom $O_2$/min/mg protein) as compared with CON (19.9 $\pm$ 4.2 ng atom $O_2$/min/mg protein). In Stage 3 of the mitochondrial respiration, oxygen consumption rates were not significantly different among groups. The values of RCI were not different among groups but P/O ratio of STZ was significantly ($p < 0.05$) increased as compared with CON (1.34 $\pm$ 0.3) and CON-AA (1.1 $\pm$ 0.3). No significant difference of those results was seen between STZ and STZ-AA.

**Table 5-1** Renal mitochondrial activity (glutamate and malate as the substrates) including respiratory control index (RCI) and ratio of amount of ADP to the oxygen utility to convert ADP to ATP (P/O) and the oxygen consumption rate of streptozotocin-induced diabetic rats and control rats with or without L-ascorbic acid supplementation at week 8 and 24 of the experimental periods (n=4).

| Oxygen consumption of renal mitochondria (Glutamate and malate as the substrates) | Groups | week 8 | week 24 |
|---|---|---|---|
| $O_2$ consumtion in Stage 4 | CON | 10.4 ± 2.0 | 7.4 ± 0.9 |
| (ng atom $O_2$/min/mg protein) | CON-AA | 9.0 ± 3.8 | 7.5 ± 1.9 [ab] |
| | STZ | 9.5 ± 4.5 | 10.4 ± 1.6 [ab] |
| | STZ-AA | 9.0 ± 3.5 | 11.3 ± 1.1 |
| $O_2$ consumtion in Stage 3 | CON | 76.6 ± 15.1 | 43.3 ± 5.1 |
| (ng atom $O_2$/min/mg protein) | CON-AA | 79.0 ± 20.3 | 36.4 ± 10.7 [ab] |
| | STZ | 66.3 ± 19.8 | 62.4 ± 12.8 [ab] |
| | STZ-AA | 54.6 ± 17.4 | 62.6 ± 8.3 |
| RCI | CON | 7.5 ± 1.9 | 5.8 ± 0.4 |
| | CON-AA | 9.5 ± 2.7 [b] | 4.9 ± 1.2 |
| | STZ | 6.3 ± 1.7 [b] | 6.0 ± 0.4 |
| | STZ-AA | 6.3 ± 1.3 | 5.5 ± 0.4 |
| P/O | CON | 1.7 ± 0.4 | 1.8 ± 0.5 [a] |
| | CON-AA | 1.8 ± 0.3 | 0.9 ± 0.4 [b] |
| | STZ | 1.9 ± 0.4 | 2.2 ± 0.5 [b] |
| | STZ-AA | 2.0 ± 0.2 | 2.0 ± 0.3 |

Mean ± SD

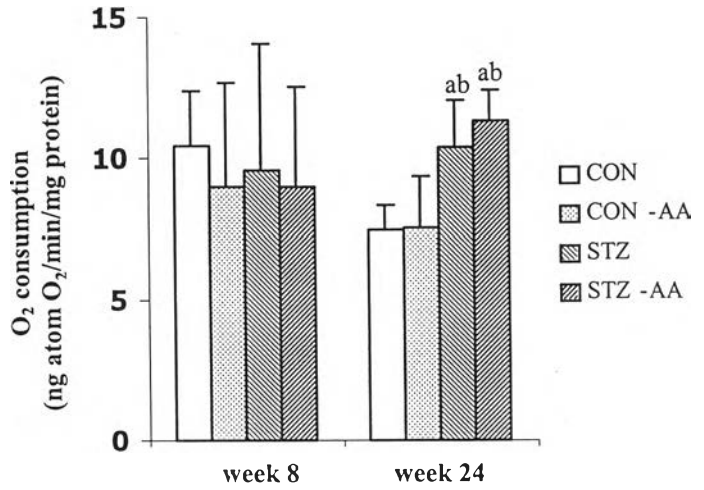
[a] compared with CON-AA and [b] compared with CON-AA at the same column, $p < 0.05$.

**Table 5-2** Renal mitochondrial activity (succinate as the substrates) including respiratory control index (RCI) and ratio of amount of ADP respected to the oxygen utility to convert ADP to ATP (P/O) and the oxygen consumption rate of streptozotocin-induced diabetic rats and control rats with or without L-ascorbic acid supplementation at week 8 and 24 of the experimental periods (n=4).

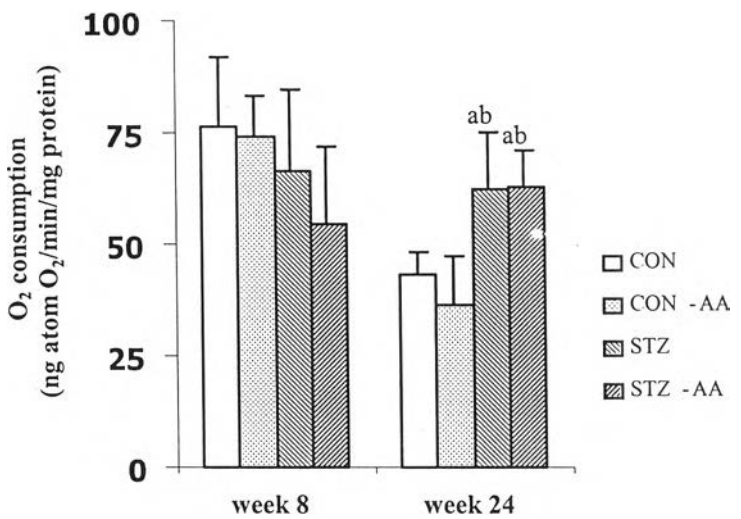
| **Renal mitochondrial activity (succinate as the substrates)** | **Groups** | **week 8** | **week 24** |
|---|---|---|---|
| **$O_2$ consumtion in Stage 4** | **CON** | 26.2 ± 5.2 | 19.9 ± 4.2 |
| **(ng atom $O_2$/min/mg protein)** | **CON-AA** | 26.8 ± 6.1 | 22.3 ± 4.4 |
| | **STZ** | 27.6 ± 7.0 | 25.8 ± 4.7 [a] |
| | **STZ-AA** | 30.0 ± 7.4 | 28.1 ± 1.6 |
| **$O_2$ consumtion in Stage 3** | **CON** | 120.0 ± 14.8 | 100.2 ± 15.7 |
| **(ng atom $O_2$/min/mg protein)** | **CON-AA** | 118.4 ± 82.7 | 105.7 ± 19.0 |
| | **STZ** | 122.5 ± 51.3 | 127.4 ± 25.3 |
| | **STZ-AA** | 125.4 ± 41.3 | 125.0 ± 10.1 |
| **RCI** | **CON** | 4.7 ± 0.8 | 5.2 ± 0.9 |
| | **CON-AA** | 3.5 ± 2.9 | 4.8 ± 0.5 |
| | **STZ** | 4.5 ± 0.9 | 5.0 ± 0.8 |
| | **STZ-AA** | 4.6 ± 1.4 | 4.5 ± 0.6 |
| **P/O** | **CON** | 1.6 ± 0.5 | 1.3 ± 0 .3 |
| | **CON-AA** | 1.3 ± 0.4 | 1.1 ± 0.3 |
| | **STZ** | 1.3 ± 0.2 | 1.7 ± 0.2 [ab] |
| | **STZ-AA** | 1.8 ± 0.6 | 1.5 ± 0.2 |

Mean ± SD

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




Stage 4




Stage 3

**Figure 5-1** Alterations of rates of oxygen consumption in stage 4 (resting stage) and stage 3 of renal mitochondrial respiration when glutamate and malate were the substrates in streptozotocin-induced diabetic rats and control rats with or without AA supplementation at week 8 and 24 of the experimental periods. All values are means $\pm$ SD. Statistically significant differences are indicated by [a] compared with CON and [b] compared with CON-AA at each period, $p < 0.05$.

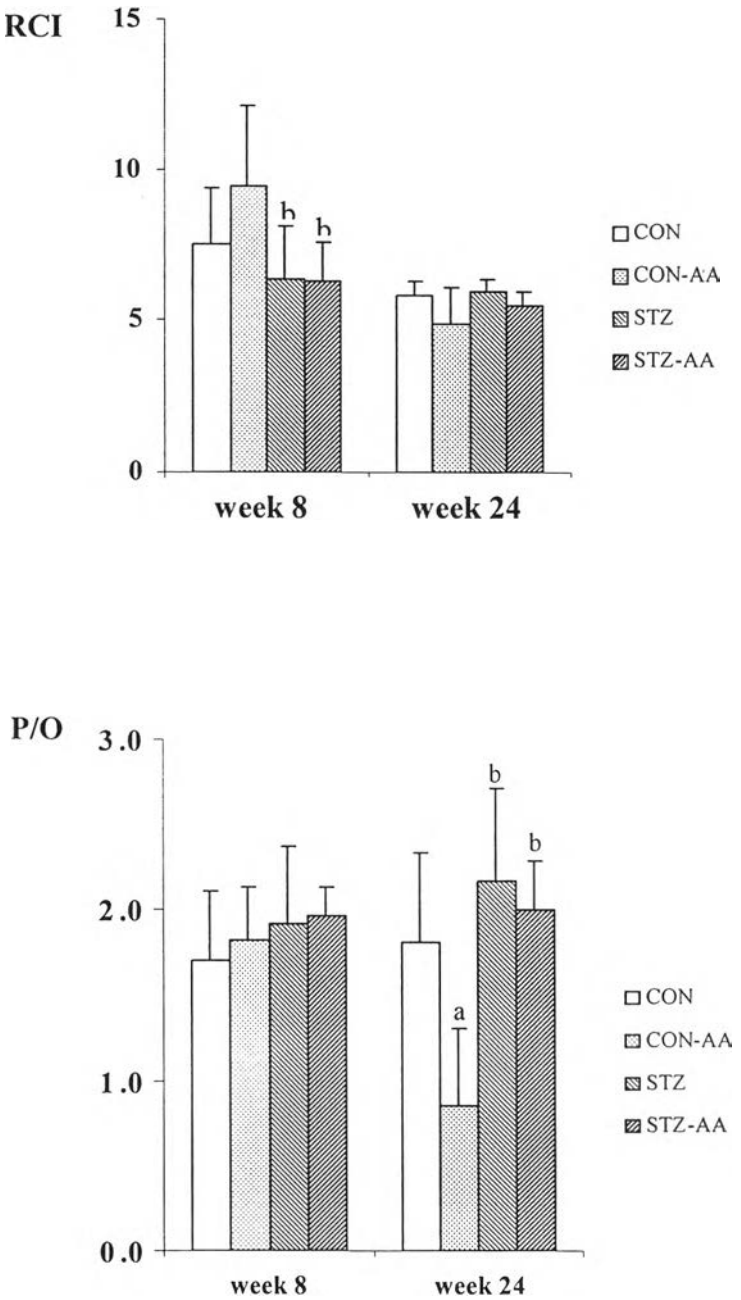
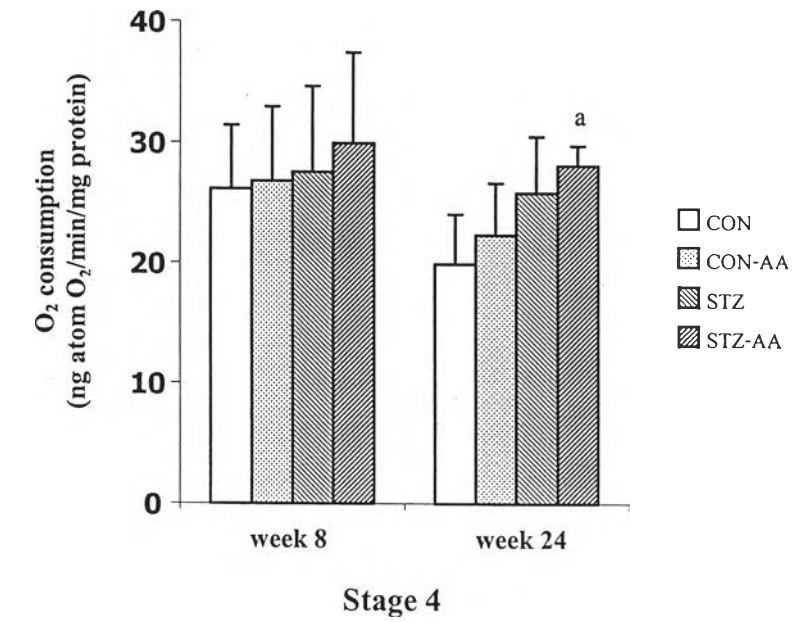
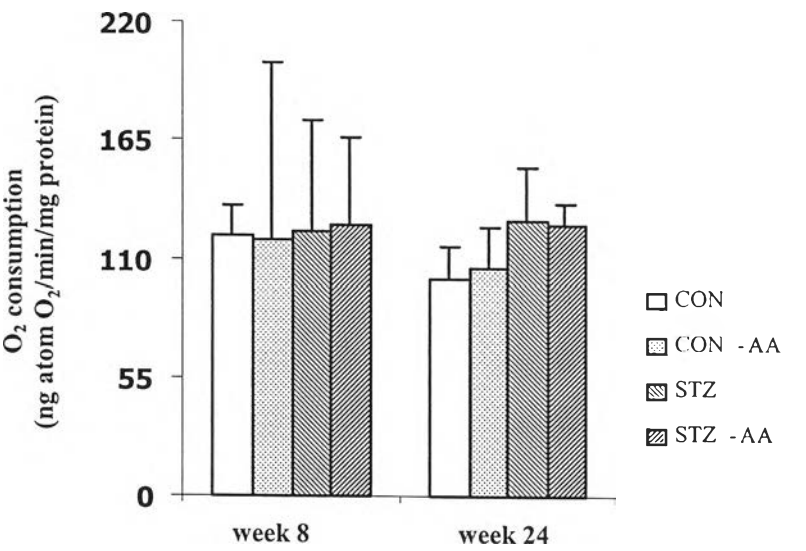



**Figure 5-2** Alterations of respiratory control index (RCI) and the ratio of amount of ADP to total oxygen consumption during stage 3 of renal mitochondrial respiration when glutamate and malate were the substrates in streptozotocin-induced diabetic rats and control rats with or without L-ascorbic acid supplementation at week 8 and 24 of the experimental periods. All values are means ± SD. Statistically significant differences are indicated by [a] compared with CON and [b] compared with CON-AA at each period, $p < 0.05$.




Stage 4




Stage 3

**Figure 5-3** Alterations of rates of oxygen consumption in stage 4 (resting stage) and stage 3 of renal mitochondrial respiration when succinate was the substrate in streptozotocin-induced diabetic rats and control rats with or without AA supplementation at week 8 and 24 of the experimental periods. All values are means ± SD. Statistically significant differences are indicated by [a] compared with CON and [b] compared with CON-AA at each period, $p < 0.05$.

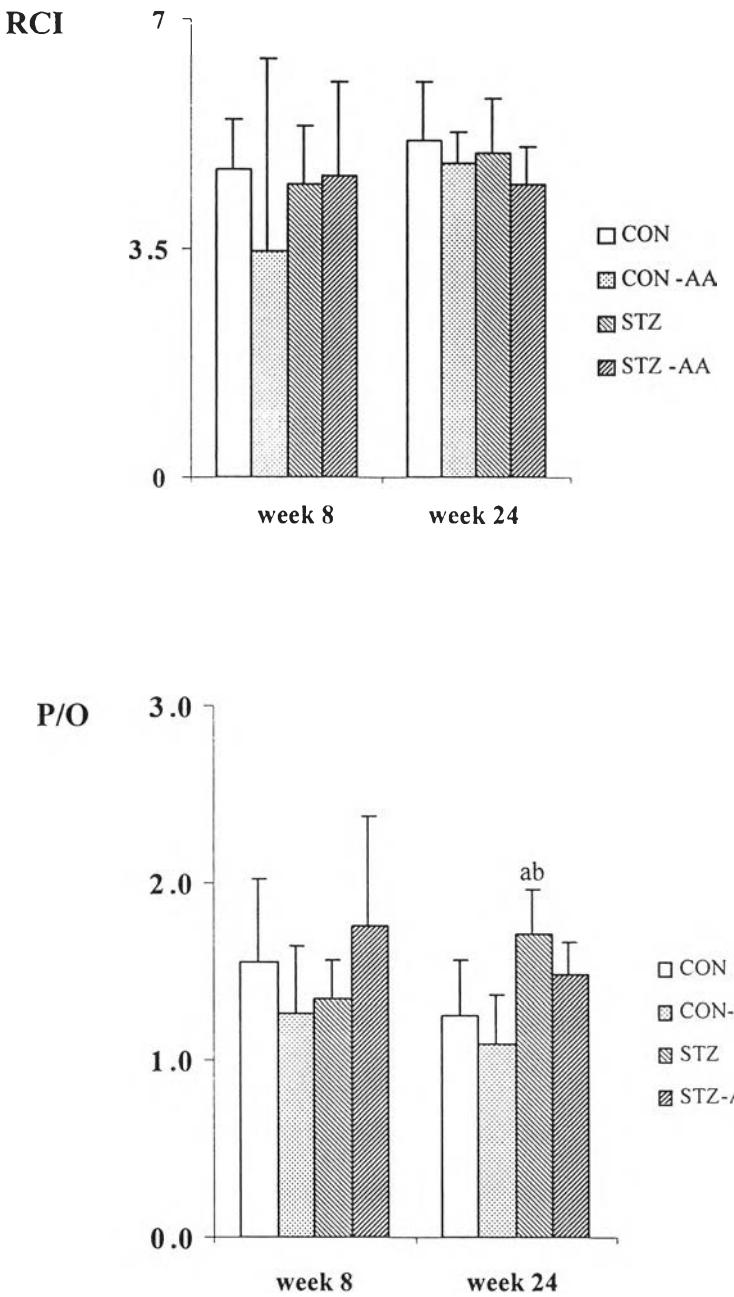



**Figure 5-4** Alterations of respiratory control index (RCI) and the ratio of amount of ADP to total oxygen consumption during stage 3 of renal mitochondrial respiration when succinate was the substrate in streptozotocin-induced diabetic rats and control rats with or without L-ascorbic acid supplementation at week 8 and 24 of the experimental periods. All values are means ± SD. Statistically significant differences are indicated by [a] compared with CON and [b] compared with CON-AA at each period, $p < 0.05$.

## Discussion

The present study was performed to investigate whether AA supplementation could ameliorate renal dysfunction in STZ-induced diabetic rats. In the present study the activity of renal mitochondria were demonstrated in two sites of the electron transport chain. Glutamate and malate are the substrates of site I and succinate is the substrate of site II. The present results shows that the RCI of the diabetic mitochondrias are good as the control mitochondria whether glutamate plus malate or succinate as the substrates of mitochondrial respiration. There was no significant difference of the mitochondrial activity (P/O) at week 8 (Table 5-1 and 5-2). This result of RCI agrees with the previous study with no alteration of renal and liver mitochondrial activity at 5 weeks of diabetes in strptozotocin-induced diabetic rats (Rogers et al., 1986). However, it was found some different results at week 24. In the case of glutamate and malate as the substrate, although the mitochondria of STZ and STZ-AA had higher rates of the oxygen consumption than that of CON in both Stage 3 and 4, P/O ratios were not markedly increased as compared with CON at week 24. It indicates that the increase in oxygen consumption might not be the increase in the phosphorylation of ADP; otherwise, there was partially uncoupling electron transport in diabetic rats. The increase in the oxidation of the substrates may occur to keep the electrochemical gradient or protonmotive force to maintain the electron transport chain. Therefore, the increase in oxygen consumption at the resting stage (Stage 4) was apparent despite of the absence of ADP. Total oxygen consumed in Stage 3 might not respond for the phosphorylation of ADP but some of oxygen consumed might be use for the maintaining the electrochemical gradient of $H^+$ between mitochondrial matrix and intermembrane space. The oxygen was rapidly consumed to oxidize the substrate but not for the phophorylatation of ADP (Lehninger, 1975). In the situation, although the mitochondria can consume the oxygen for the substrate oxidation with the higher rate than normal, not only ATP is not synthesized but ATP will be hydrolyzed (Avers, 1986). This finding indicates the uncoupled mitochondrial respiration in site I of the diabetic rats. The unchanging in P/O (as compared with that of CON) with the increase in the oxygen consumption in the activated stage (Stage 3) indicated that the oxygen was consumed in order to oxidize the substrate to keep the electron transport chain and to maintain the proton motive force. Therefore the mitochondrial dysfunction in site I would occur in the

electrochemical gradient of proton rather than NADH dehydrogenase (complex I) or ATPase activity. The persistence of P/O ratio may indicate the ATPase acitivity which is possibly normal to perform the phosphorylation of ADP. It is confirmed by the result of an increase in P/O ratio when succinate was used as the substrate in the present study. However, the histochemical study of the previous study indicated the decrease in ATPase activity in diabetic rats (Stefek et al., 2002).

In the case of succinate as the substrate, the oxygen consumption rate in Stage 3 and 4 of the diabetic groups seems to be increased as compared with that of CON but only STZ-AA was significantly increased ($p < 0.05$) in the rate of oxygen consumption rate in Stage 4. Base on the property of ascorbate as one of the substrate that can directly transport the electron to cytochrome c, it may be explained by the presence of oxidation of ascorbate in the intact mitochondria. In the present result, the value of P/O of STZ was significantly increased ($p < 0.05$) as compared with CON and CON-AA. It indicates that the increase in mitochondrial respiration occured in site II. These changes might be caused by either the property of succinate to directly transport electrons to coenzyme Q, resulting in maintaining electrochemical gradient of proton between matrix and intermembrane space of mitochondria or the possible increase in the activities of succinate dehydrogenase and ATPase in diabetic rats. In contrast with the histochemical study, the decrease in succinate dehydrogenase activity was noted (Stefek et al., 2002). In the present study, the value of P/O of STZ-AA was normalized. It was not significantly different to either that of CON or STZ. This is interesting for the beneficial effect of AA supplementation to ameliorate the mitchondrial activity in site II in STZ-induced diabetic rats. The mechanism of AA to normalize the mitochondrial respiration in site II is needed a further investigation.

In conclusion, the STZ-induced diabetic rats had mitochondrial dysfunction with the decrease in the activity of site I and increase in the activity of site II of mitochondrial respiration. AA supplementation has a beneficial effect of the decrease in the mitochondrial respiration in site II of the STZ-induced diabetic rats.