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Reduced activity of mtTFA decreases the transcription in mitochondria isolated from diabetic rat heart

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Kanazawa, Akio, Yoshihiko Nishio, Atsunori Kashiwagi, Hidetoshi Inagaki, Ryuichi Kikkawa, and Kihachiro Horiike. Reduced activity of mtTFA decreases the transcription in mitochondria isolated from diabetic rat heart. *Am J Physiol Endocrinol Metab* 282: E778–E785, 2002; 10.1152/ajpendo.00255.2001.—To evaluate abnormalities in the mitochondrial transcription factor A (mtTFA) function as a cause of mitochondrial dysfunction in diabetes, we measured the mRNA contents of the proteins consisting of the mitochondrial respiratory chain as well as transcriptional and translational activities in the mitochondria isolated from controls and streptozotocin-induced diabetic rat hearts. Using Northern blot analysis, we found 40% reduced mRNA contents of mitochondrial-encoded cytochrome *b* and ATP synthase subunit 6 in diabetic rat hearts compared with control rats ($P < 0.05$). These abnormalities were completely recovered by insulin treatment. Furthermore, the mitochondrial activities of transcription and translation were decreased significantly in mitochondria isolated from diabetic rats by 60% ($P < 0.01$) and 71% ($P < 0.01$), respectively, compared with control rats. The insulin treatment also completely normalized these abnormalities in diabetic rats. Consistently, gel retardation assay showed a reduced binding of mtTFA to the D-loop of mitochondrial DNA in diabetic rats, although there was no difference in the mtTFA mRNA and protein content between the two groups. On the basis of these findings, a reduced binding activity of mtTFA to the D-loop region in the hearts of diabetic rats may contribute to the decreased mitochondrial protein synthesis.

transcription factor; translation; oxidative stress; diabetes mellitus

DIABETES MELLITUS accompanies mitochondrial dysfunctions such as the abnormalities in oxidative phosphorylation (20, 31). These abnormalities cause a variety of diseases including cardiomyopathy (15) and neuromuscular diseases (37). The mitochondrial dysfunction in islet β-cells impairs insulin secretion in response to increased glucose concentration (39). Thus mitochondrial dysfunctions are deeply involved in the pathophysiology of diabetes and its complications. The interaction between mitochondrial dysfunction and oxidative stress has also been reported (16, 23). For example, it was reported that the increased electron leakage from complex I of mitochondrial electron transport resulted in increased oxidative stress in the failing heart (11). In neurodegenerative disease, such as Parkinson's disease, excessive generation of superoxide anions in mitochondria was involved in its pathophysiology (24). Moreover, we (28) previously reported elevated oxidative stress in the hearts of diabetic rats, and Nishikawa et al. (26) reported that a high glucose condition increased the production of superoxide anions in mitochondria of cultured bovine aortic endothelial cells. Thus the pathological roles of mitochondria as a source of reactive oxygen species have been very much noted.

Conversely, oxidative stress itself causes mitochondrial disorders such as functional impairment in respiration, accumulation of mutated mitochondrial DNA (37), and decreased mitochondrial gene expression (2, 14). Reduced mitochondrial function causes the accumulation of oxidative stress in the cells (3). These findings suggest that diabetes-induced oxidative stress may affect mitochondrial gene expression and its transcriptional activity.

Mitochondria contain closed, circular, double-stranded DNA of 16.5 kb in size. Both strands of the mitochondrial DNA are transcribed. Transcription of mitochondrial DNA is initiated at two different promoters, heavy- and light-strand promoters, located within the D-loop region, and requires mitochondrial transcription factor A (mtTFA) (30, 40) and RNA polymerase (7). mtTFA is the nuclear-encoded DNA binding protein containing two high-mobility group domains. Recently, Wang et al. (38) generated heart-specific mtTFA knockout mice. In this animal model, the level of mitochondrial transcription was markedly decreased, and the activity of the mitochondrial-encoded respiratory chain subunit was also decreased, suggesting that mtTFA plays a major role in mitochondrial transcription and its functions. Furthermore, it was reported that the mRNA contents of mitochondrial-encoded genes were decreased in the pancreas of

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diabetic rats (33). However, the mechanisms underlying the decreased mitochondrial gene expression in diabetes are not clear, and little is known about mitochondrial gene regulation in the diabetic heart. Indeed, the pathophysiological significance of mtTFA in the diabetic heart remains unknown.

On the basis of these observations, it is suggested that mtTFA may be a clue to elucidating the mechanisms for mitochondrial gene regulation and reduced mitochondrial function in diabetes mellitus and that diabetes-induced oxidative stress may affect mitochondrial transcriptional activity in the heart. We investigated the expression and activity of mtTFA and mitochondrial gene regulation in the hearts of diabetic rats.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 180–200 g were randomly separated into control ($n = 10$) and experimental groups (diabetic and insulin-treated diabetic rats, $n = 10$ each). The experimental animals were anesthetized with diethyl ether and given an intravenous injection of streptozotocin (50 mg/kg) in 0.05 mM citrate buffer. The onset of diabetes was determined by measuring blood glucose levels. These animals were maintained on a laboratory diet and water ad libitum for 4 wk after the streptozotocin injection, and all measurements in the present study were taken 4 wk after streptozotocin injection. Insulin pellets (Linshin, Scarborough, Canada) were implanted subcutaneously 3 days after the streptozotocin injection to normalize blood glucose levels.

Northern blot analysis. Total RNA was extracted from the cardiac ventricle by the acid guanidinium thiocyanate-phenol-chloroform method (4). Total RNA (10 μg) was fractionated by denaturing 1% formaldehyde agarose gel electrophoresis and transferred to Nytran (Schleicher & Schuell, Keene, NH) and cross-linked by ultraviolet irradiation. Hybridization and washing of the membrane were carried out with α-^{32}P-labeled complementary DNA (cDNA) probes for rat ATP synthase subunit 6, cytochrome *b*, cytochrome *c*, and mtTFA, as reported previously (28). The signal intensity was estimated by Molecular Imager System (Bio-Rad Laboratories, Hercules, CA). To normalize the loading differences, the same membrane was rehybridized with 36B4 (human acidic ribosomal phosphoprotein) cDNA. Rat cDNAs of cytochrome *b*, cytochrome *c*, ATP synthase subunit 6, and mtTFA were cloned by reverse transcription-polymerase chain reaction with the use of the mRNA isolated from a rat heart with the following synthetic oligomers: cytochrome *b*, 5′-TCTCATCAGTCACCCACATC-3′ and 5′-CATTCTGGTTTGATGTGGGG-3′; cytochrome *c*, 5′-ATGGGTGATGTTGAAAAAAGG-3′ and 5′-TTATTCATTAGTAGCCTTTT-3′; ATP synthase subunit 6, 5′-CATCAGAACGCCTAATCAGC-3′ and 5′-GTAGGTACAGGCTGACTAGA-3′; mtTFA, 5′-AGCAAATGGCTGAAGTTGGG-3′ and 5′-TCTAGTAAAGCCCGGAAGGT-3′.

In vitro transcription assay. Mitochondria were isolated from rat hearts by the protease method (22), and the integrity of isolated mitochondria was confirmed by evaluating respiratory control ratio (>4.5) and ADP/O ratio (1.8), which were measured using an oxygen electrode with 10 mM succinate as the substrate. The activity of mitochondrial transcription was measured as reported previously (6, 9). In brief, the reaction mixture contained isolated mitochondria (10 mg protein/ml), 40 mM Tris·HCl (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, 2 mg/ml bovine serum albumin, 20% (vol/vol) glycerol, 10 mM Na_2HPO_4, 0.2 mg/ml creatine kinase, 2 mM ATP, 1 mM CTP, 1 mM GTP, 5 mM creatine phosphate, and 4 mM pyruvate in a total volume of 50 μl. The reaction was started by adding [α-^{32}P]UTP (29.6 TBq/mmol, New England Nuclear, Boston, MA) at a concentration of 7.4 MBq/ml, and incubation was at 37°C for 30 min. An aliquot of the reaction mixture corresponding to 5 μl was mixed with 5 μl of 5% (wt/vol) sodium dodecyl sulfate (SDS) solution at 10-min intervals and spotted on DE81 filter paper (Whatman), and the filter paper was washed three times in washing buffer containing 100 mM sodium phosphate (pH 7.4) and 200 mM NaCl and transferred to scintillation vials. To estimate the background radioactivity, 0.1 mg/ml ethidium bromide was added to the samples of control, diabetic, and insulin-treated diabetic rats. The radioactivity was measured using a Packard liquid scintillation counter. To investigate the effects of oxidative stress and thyroid hormone on the mitochondrial transcription, hydrogen peroxide (0.5, 1, and 2 mM) and triiodothyronine (T_3; 500 pg/ml) were added to the incubation medium containing mitochondria isolated from control and diabetic rat hearts.

Electrophoretic gel shift assay. A radioactive probe containing the nucleotide sequence of the heavy-strand promoter was prepared by annealing paired oligonucleotides with the sequences 5′-TTTCCTCCTAACTAAACCCTCTTTAC-3′ and 5′-GTAGGCAAGTAAAGAGGGTTTAGTTA-3′ and was labeled using [α-^{32}P]dATP (New England Nuclear) and DNA polymerase (Takara, Shiga, Japan). The protein-DNA binding protein reaction was performed at room temperature for 20 min in a volume of 20 μl. The reaction mixture contained 10 μg of mitochondrial extract protein (34), 100 μg/ml poly dI:dC, 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, 4% glycerol, and 100,000 cpm labeled oligonucleotides. After the incubation, samples were loaded onto 8% polyacrylamide gels in 0.25× Tris-borate-EDTA buffer and run at 150 V for 2 h. The gels were dried, and the bands were visualized by autoradiography. For competition assay, nonlabeled oligonucleotides were added at a 50-fold molar excess to the reaction mixture before the addition of mitochondrial extract protein.

Western blot analysis of mtTFA. Anti-rat mtTFA serum was prepared as described previously (12). For Western blotting, total heart homogenate and isolated mitochondria were suspended in ice-cold lysis buffer containing 20 mM Tris·HCl (pH 7.5), 50 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 μM aprotinin, 5 μg/ml leupeptin, and 2 mM benzamidine. After centrifugation at 17,000 *g* at 4°C for 20 min, the supernatant (30 μg of protein) was resolved on 12% SDS-polyacrylamide gel, electrotransferred to an Immobilon P membrane (Millipore, Bedford, MA), and blotted with anti-rat mtTFA serum. Bound antibodies were detected with horseradish peroxidase-conjugated anti-IgG and visualized with an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech).

In vitro translation assay. Mitochondrial protein synthesis was measured by the method of McKee et al. (21). In brief, mitochondrial protein (4 mg/ml) was suspended in a volume of 100-μl protein synthesis medium (pH 7.0) containing 20 mM glutamate, 0.5 mM malate, 44 mM mannitol, 14 mM sucrose, 25 mM MOPS, 90 mM KCl, 2.5 mM KH_2PO_4, 0.4 mM EGTA, 4 mM $MgSO_4$, 2 mM ADP, 0.1 mg/ml cyclohexamide, 1 mg/ml bovine serum albumin, and 0.5 mM of L-amino acids except methionine. After preincubation at 30°C for 5 min, 20 μM methionine ([^{35}S]methionine, 37.0 TBq/mmol, 7.4 MBq/ml, New England Nuclear) were added, and 10-μl aliquots were sequentially removed at 0, 10, 20, and 30 min

after the addition of [^{35}S]methionine and spotted on Whatman 541 filter paper. The filter was washed three times in 5% (wt/vol) trichloroacetic acid containing 5 mM methionine and transferred to scintillation vials. The radioactivity was measured after the addition of 10 ml of scintillation fluid by use of a Packard liquid scintillation counter.

Hydrogen peroxide production in mitochondria isolated from hearts. Hydrogen peroxide production was determined by the horseradish peroxidase (HRP)-dependent reaction of hydrogen peroxide with the fluorescent dye scopoletine (18). Rat heart mitochondria (200 μg) were added to a cuvette containing 2 ml of air-saturated respiration buffer [0.25 M sucrose, 10 mM Tris·HCl (pH 7.4), 5 mM K_2HPO_4, 1 mM $MgCl_2$]. After addition of the scopoletine (1 μM) and HRP (0.2 μM), the fluorescence measurements were carried out in a Spectro-Fluorometer (excitation 365 nm, emission 450 nm), and a decrease in fluorescence intensity indicated an oxidation of scopoletine by hydrogen peroxide via HRP. A standard curve was obtained using the previously known concentrations of hydrogen peroxide.

Measurement of lipid peroxide contents in mitochondria isolated from hearts. The lipid peroxide contents of mitochondria were measured in a previous report (1). In brief, the lipid fraction-isolated mitochondria were extracted with a chloroform-methanol solution. The lipid fraction was resuspended in 100 μl of methanol with or without 10 mM triphenylphosphine, and 900 μl of FOXII reagent (29) were added. The difference in absorbance at 560 nm between the samples with and without TTP was considered the lipid peroxide content. A standard curve was obtained using the previously known concentrations of hydrogen peroxide.

Protein assay. Protein content was determined by the method of Lowry (19), with bovine serum albumin as a standard.

Statistical analysis. All values are presented as means ± SE. Statistical comparison of means among individual groups was performed using analysis of variance (ANOVA) followed by post hoc testing with Fisher's least significant difference test. P values <0.05 were considered significant.

RESULTS

Animal characteristics. Diabetic rats showed significantly higher plasma glucose levels (28.74 ± 1.22 vs. 9.06 ± 0.31 mM, $P < 0.01$) and lower body weights (170.5 ± 9.4 vs. 356.5 ± 7.2 g, $P < 0.01$) and plasma insulin levels (27.0 ± 2.4 vs. 144.0 ± 19.2 pM, $P < 0.05$) compared with control rats. The levels of plasma glucose were not changed between control and insulin-treated diabetic rats (7.60 ± 1.20 mM), whereas body weights in the insulin-treated diabetic rats were significantly lower than those of control rats (323.0 ± 6.4 g, $P < 0.05$), and plasma insulin levels were significantly higher than those of control rats (297.6 ± 63.0 pM, $P < 0.01$).

mRNA contents of ATP synthase subunit 6 and cytochrome b in the hearts of diabetic rats. The mRNA contents of ATP synthase subunit 6 and cytochrome *b* were determined in the hearts of control and diabetic rats by means of Northern blot analysis. As shown in Fig. 1*A*, we could detect the precursor and mature forms of the mRNA of ATP synthase subunit 6 and cytochrome *b*. These mRNA contents were significantly decreased in the hearts of diabetic rats. The insulin treatment prevented the diabetes-induced decrease in these mRNA contents in the hearts. Figure 1*B* shows the results of the quantitation of the Northern blot analysis. The mature mRNA contents of ATP synthase subunit 6 and cytochrome *b* were significantly decreased by 40% ($P < 0.05$).

mRNA contents of mtTFA and cytochrome c in the hearts of diabetic rats. Figure 2*A* presents the results of Northern blot analysis of the mtTFA and cytochrome *c* in the hearts of diabetic rats. The mRNA contents of the mtTFA and cytochrome *c* in diabetic rats were not different from those of control rats, and quantitative analysis showed that the mRNA contents of mtTFA

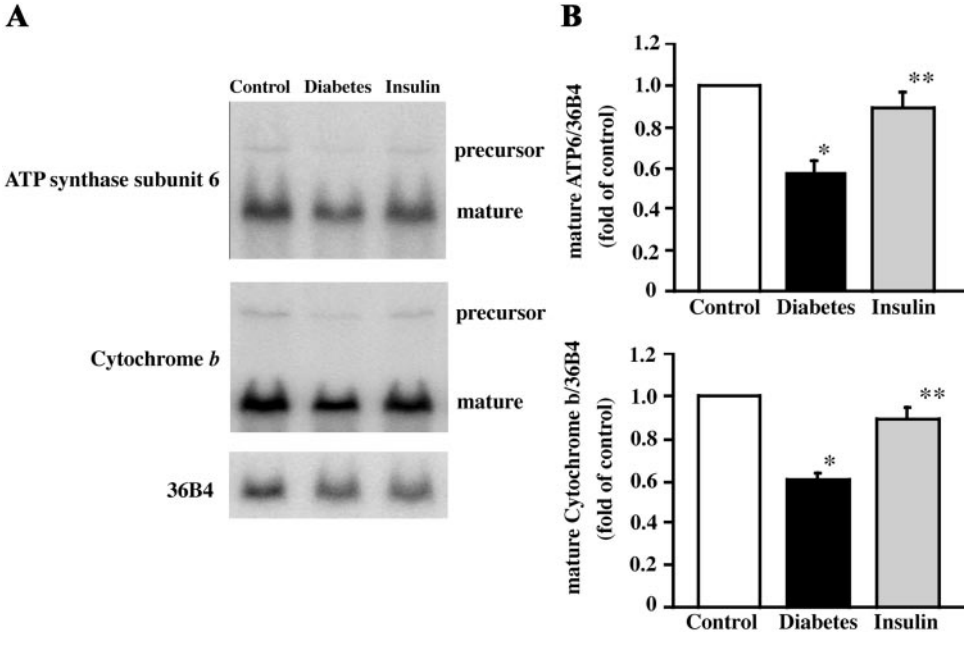

Fig. 1. *A*: Northern blot analysis of ATP synthase subunit 6 and cytochrome *b* by use of 10 μg of total RNA isolated from the hearts of control and diabetic rats without (Diabetes) or with insulin treatment (Insulin). The same blot was reprobed with 36B4 cDNA as a loading control. *B*: quantification of the ATP synthase subunit 6 and cytochrome *b* contents detected by Northern blot analysis in the hearts of control ($n = 6$) and diabetic rats without ($n = 6$) or with insulin treatment ($n = 6$). 36B4, a human acidic ribosomal phosphoprotein. Values are means ± SE. *$P < 0.05$ vs. Control; **$P < 0.01$ vs. Diabetes.

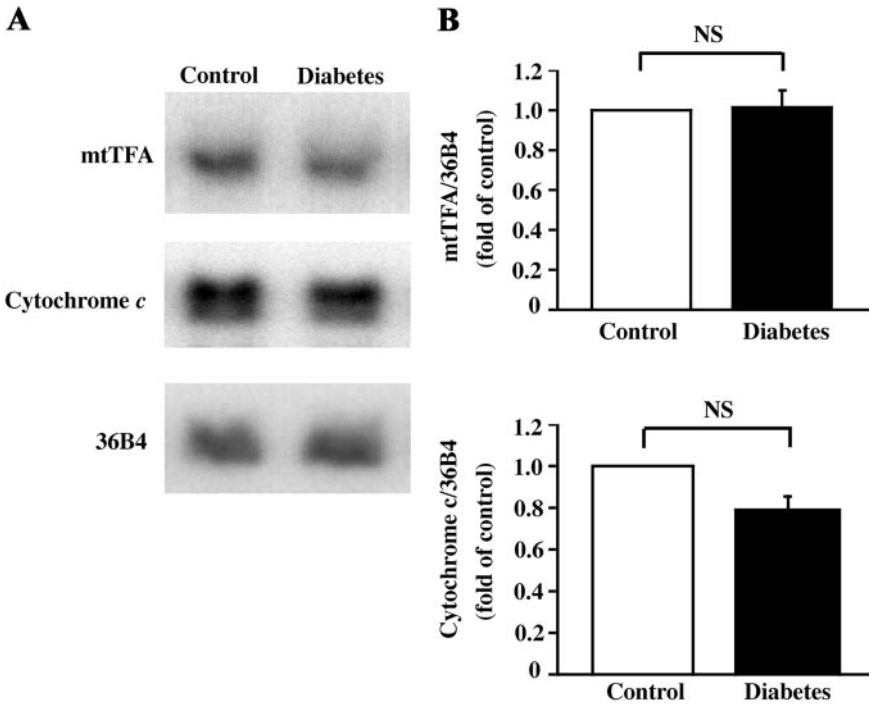

Fig. 2. *A*: Northern blot analysis of mitochondrial transcription factor A (mtTFA) and cytochrome *c* by use of 30 μg of total RNA isolated from the hearts of control (n = 3) and diabetic rats (n = 3). The same blot was reprobed with 36B4 cDNA as a loading control. *B*: quantification of mtTFA and cytochrome *c* contents detected by Northern blot analysis in the hearts of control and diabetic rats. NS, not significant. Values are means ± SE.

and cytochrome *c* were not different between control and diabetic rats (Fig. 2*B*).

Protein contents of mtTFA in the hearts of diabetic rats. Western blot analysis of the mtTFA demonstrated that there was no significant difference in the mtTFA protein contents in the total heart homogenate and isolated mitochondria between control and diabetic rats (Fig. 3, *A* and *B*).

In vitro transcription assay in the hearts of diabetic rats. Figure 4 shows the results of transcriptional activity determined by measuring the fractional incorporation of [α-^{32}P]UTP into mitochondrial RNA. The transcriptional activity of mitochondria in the diabetic rats was significantly decreased during the 30-min assay period, and the insulin treatment completely prevented the diabetes-induced decrease in the transcriptional activity. In addition, the UTP transport rate into the mitochondria using either 0.25 μM (0.19 ± vs. 0.15 ± 0.02 pmol/mg protein, n = 3, respectively) or 250 μM UTP (276.7 ± vs. 253.7 ± pmol/mg protein, n = 4, respectively) at 37°C for 30 min was not different between control and diabetic rats, respectively.

Binding activity of mtTFA to the D-loop region in the hearts of diabetic rats. The binding activity of the α-^{32}P-labeled oligonucleotide containing the heavy-

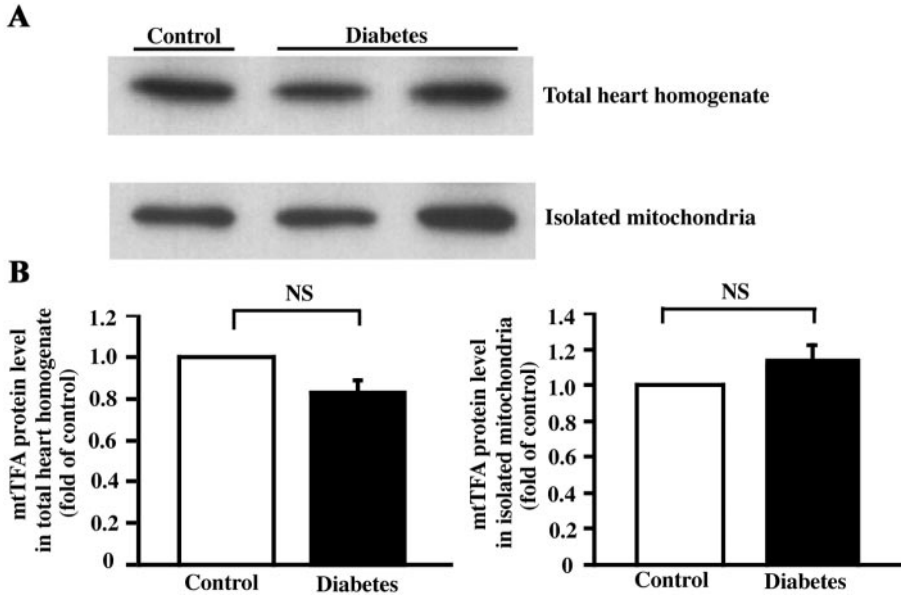

Fig. 3. *A*: Western blot analysis of mtTFA in the total heart homogenate and isolated mitochondria from the hearts of control (n = 4) and diabetic rats (n = 5). *B*: protein contents of mtTFA were quantified with a densitometer. Values are means ± SE.

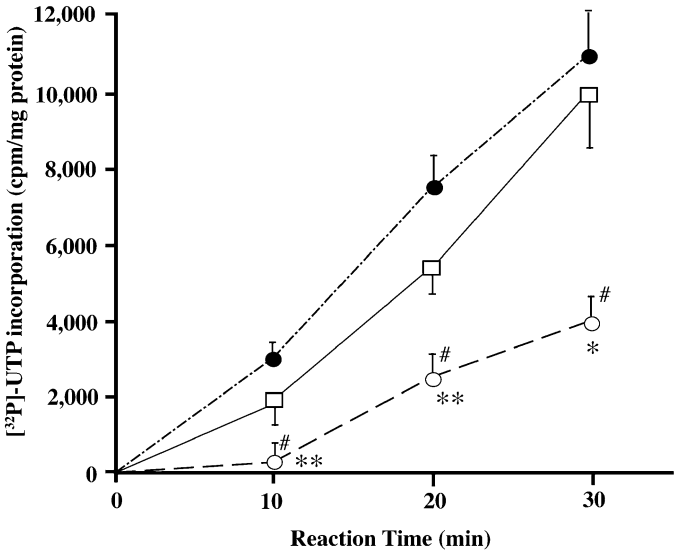

Fig. 4. In vitro transcription assay of mitochondria isolated from the hearts of control (□, $n = 4$) and diabetic rats without (Diabetes: ○, $n = 4$) or with insulin treatment (Insulin: ●, $n = 4$). Values are means ± SE. Background radioactivity at 30 min in control (3,380 ± 312 cpm/mg protein), diabetic (2,960 ± 150 cpm/mg protein), and insulin-treated diabetic rats (3,172 ± 214 cpm/mg protein). The background counts were subtracted from the data. $^{*}P < 0.01$ and $^{**}P < 0.05$ vs. Control; $^{\#}P < 0.01$ vs. Insulin.

strand promoter sequence within the D-loop to mitochondrial extracted protein from the hearts of control and diabetic rats was investigated using an electrophoretic gel mobility shift assay. The specificity of the shifted band was confirmed by the finding that the band disappeared in the presence of a 50-fold molar excess of unlabeled oligonucleotide. As shown in Fig. 5*A*, the binding activity of mtTFA in the hearts of diabetic rats to oligonucleotide containing the heavy-strand promoter sequence was decreased. The insulin treatment prevented the decrease in the binding activity of mtTFA in diabetes. Figure 5*B* shows the results of the quantification of the binding activity of mtTFA. The binding activity of mtTFA in the diabetic rats was significantly decreased by 47% ($P < 0.05$), and there was no difference between control and insulin-treated diabetic rats.

In vitro translation assay in the hearts of diabetic rats. The synthesis of mitochondrial protein was assessed by measuring the fractional incorporation of [^{35}S]methionine into mitochondrial protein. As shown in Fig. 6, the translational activity of mitochondrial protein in diabetic rats was significantly lower than that of control rats at 30 min by 71% ($P < 0.01$), and the insulin treatment completely prevented the diabetes-induced decrease in the translational activity.

Effect of thyroid hormone to the mitochondria isolated from diabetic rat hearts. Transcriptional activity in mitochondria isolated from diabetic rat hearts at 20 min (18,932 ± 3,380 cpm/mg protein, $n = 3$) did not change significantly by direct addition of T_3 (17,166 ± 2,466 cpm/mg protein, $n = 3$).

Basal production of hydrogen peroxide and lipid peroxide contents in the mitochondria isolated from diabetic rats. As shown in Fig. 7*A*, basal production of hydrogen peroxide in the mitochondria isolated from diabetic rats was significantly increased 2.3-fold ($P < 0.01$). The lipid peroxide contents of mitochondria isolated from diabetic hearts were significantly increased 4.5-fold ($P < 0.01$). Insulin treatment completely reversed the increased lipid peroxide contents in the mitochondria isolated from diabetic rats (Fig. 7*B*).

Effect of hydrogen peroxide on the mitochondrial transcription. Exposure of isolated mitochondria to hydrogen peroxide (0.5 and 2 mM) resulted in a dose-dependent decrease in mitochondrial transcription (Fig. 8). A lower dose of hydrogen peroxide (0.5 mM)

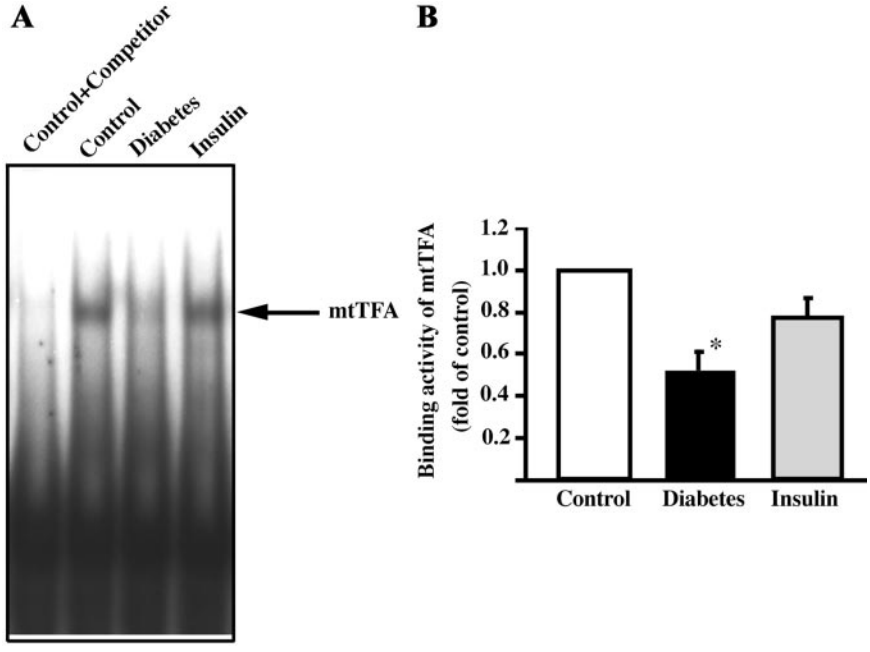

Fig. 5. *A*: binding activity of mtTFA to the D-loop region in the hearts of control ($n = 4$) and diabetic rats without (Diabetes, $n = 4$) or with insulin treatment (Insulin, $n = 4$). Mitochondrial extract protein (10 μg) was incubated with α-^{32}P-labeled heavy-strand promoter probe. The mixture was electrophoresed in 8% polyacrylamide gel, dried, and exposed to X-ray film. *B*: quantification of the binding activity of mtTFA detected by electrophoretic mobility shift assay in the hearts of control ($n = 3$) and diabetic rats without (Diabetes, $n = 4$) or with insulin treatment (Insulin, $n = 4$).

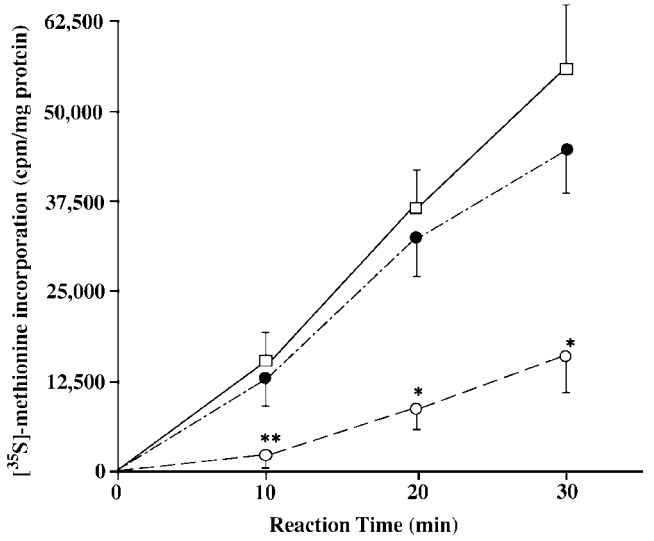

Fig. 6. In vitro translation assay of mitochondria isolated from the hearts of control (□, $n = 4$), diabetic (○, $n = 4$), and insulin-treated diabetic rats (●, $n = 3$). Mitochondria (4 mg/ml) were incubated with [35S]methionine in a translation incubation mixture. Values are means ± SE. Background radioactivity at 0 min in control (10,631 ± 672 cpm/mg protein), diabetic (11,343 ± 640 cpm/mg protein), and insulin-treated diabetic rats (10,494 ± 170 cpm/mg protein). The background counts were subtracted from the data. *$P < 0.01$ and **$P < 0.05$ vs. Control.

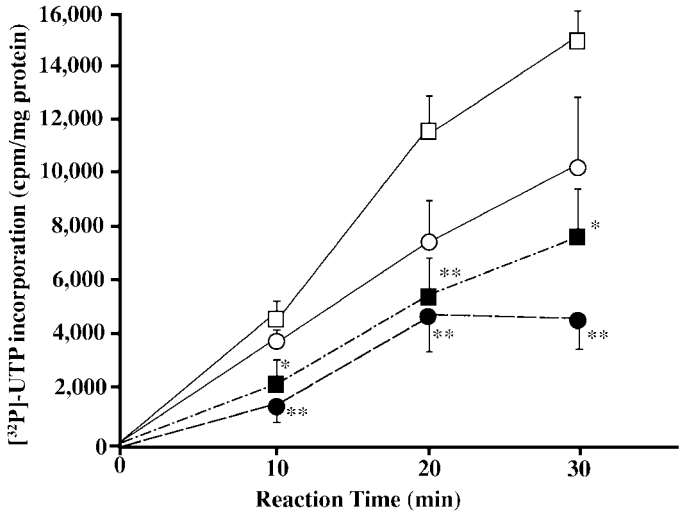

Fig. 8. Effects of hydrogen peroxide on mitochondrial transcription. Isolated mitochondria were treated for 30 min with 0.5 mM (○, $n = 5$), 1 mM (■, $n = 5$), and 2 mM (●, $n = 5$) hydrogen peroxide. Values are means ± SE. Background radioactivity at 30 min in control was 4,133 ± 240 cpm/mg protein. The background counts were subtracted from the data. *$P < 0.05$ and **$P < 0.01$ vs. Control (□, $n = 5$).

showed a decreased tendency in mitochondrial transcription; however, this was not significant.

DISCUSSION

The present study demonstrated that the mRNA contents of mitochondrial-encoded ATP synthase subunit 6 and cytochrome *b* in the hearts of streptozotocin-induced diabetic rats were decreased, whereas those of nuclear-encoded cytochrome *c* and mtTFA were not decreased. Consistent with these findings, the reduced binding activity of the mtTFA and the decreased transcriptional activity of mitochondria were found in the hearts of diabetic rats. Insulin treatment completely normalized these abnormalities in streptozotocin-induced diabetic rats, indicating that these changes were the result of the diabetic state and not a toxic effect of streptozotocin itself.

Recently, some studies have reported that mtTFA plays a critical role in the regulation of mitochondrial gene expression (13, 25). The mtTFA, which is nuclear-encoded proteins, is imported to the mitochondrial matrix and activates the mitochondrial transcription by binding to the promoter region within the D-loop of mitochondrial DNA. To our knowledge, the expression and binding activity of mtTFA in the hearts of diabetic rats have not been reported. Therefore, to clarify the mechanism of decreased mitochondrial transcriptional activity in diabetic hearts, Western blot analysis of mtTFA and the binding of mitochondrial protein to the specific binding site within the D-loop region were performed. The mRNA and protein contents of mtTFA in control and diabetic rats were not significantly different. However, the binding activity of mtTFA in diabetic rat hearts was significantly decreased in the present study. These findings suggest that an impairment of mtTFA binding to D-loop of mitochondrial DNA may be explained by the abnormalities at the regulatory sites, such as mtTFA transport into mitochondria, modification of mtTFA protein, and various mutations of mitochondrial DNA in diabetes.

In the present study, we confirmed that mtTFA transport into mitochondria was not impaired. However, besides the mtTFA, RNA polymerase is needed

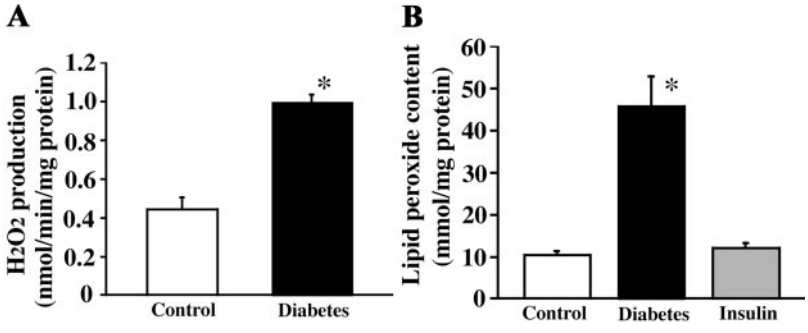

Fig. 7. *A*: basal production of hydrogen peroxide in mitochondria isolated from the hearts of control ($n = 3$) and diabetic rats ($n = 3$). *$P < 0.01$ vs. Control. *B*: lipid peroxide contents of mitochondria isolated from control ($n = 4$) and diabetic rats without (Diabetes, $n = 4$) or with insulin treatment (Insulin, $n = 3$). Values are means ± SE. *$P < 0.01$ vs. Control and Insulin.

sfor the transcriptional process. Because the activity of RNA polymerase was not measured in this study, we cannot conclude that the reduced binding activity of mtTFA was the sole cause of the decreased transcriptional activity in diabetic rats. However, mtTFA has the ability to wrap and unwind DNA through formation of the protein-DNA complex (8). These mtTFA-induced conformational changes of mitochondrial DNA may be required to allow the RNA polymerase access to the template for initiation of the transcription process. Therefore, a reduced function of mtTFA may result in insufficient RNA polymerase access to the initiation site of the transcription and may account for the decrease in mitochondrial transcriptional activity in diabetic hearts.

In relation to oxidative modification of mtTFA protein in diabetes, we found an increased basal production of hydrogen peroxide and an increased lipid peroxide content in the mitochondria isolated from diabetic rat hearts. Furthermore, hydrogen peroxide treatment on the mitochondria isolated from the rat hearts decreased its transcriptional activity. These findings may suggest that mtTFA exposed to the elevated oxidative stress induced by diabetes is accompanied by abnormal modifications such as protein oxidation. Furthermore, the mitochondrial antioxidant systems, including gluthatione peroxidase, may reduce the concentration of hydrogen peroxide inside the mitochondria during incubation. Therefore, although we exposed 0.5–2.0 mM hydrogen peroxide to isolated mitochondria in this present study, it was very difficult to measure the exact intramitochondrial hydrogen peroxide concentration. Thus the physiological relevance of this relatively high hydrogen peroxide-induced impairment of transcriptional activity in mitochondria should be further evaluated in the future.

Moreover, we should note the possibility that a mutation in the D-loop region of mitochondrial DNA contributes to the decreased transcriptional activity, since it has been reported by Takeda et al. (36) that isolated mitochondria from diabetes accumulate from a mutated DNA due to the elevated oxidative stress.

It is noted that, as a possible mechanism for decreased mitochondrial transcription in diabetes, hypothyroidism is a cause of decreased mRNA contents of mitochondrial-encoded genes (35). Serum T_3 levels are often depressed in clinical (32) and experimental diabetes (10). T_3 directly regulates mitochondrial RNA synthesis through T_3 receptor in the mitochondrial matrix (5). Therefore, it is possible that diabetes-induced impairment of mitochondrial transcriptional activity in isolated mitochondria is due, in part, to a deficiency of T_3. However, we previously reported that there were no differences in serum T_3 levels between controls and 10-wk-diabetic rats (27), indicating that chronic diabetes did not exhibit a severe hypothyroidism. Furthermore, direct addition of T_3 to the incubation medium of mitochondria isolated from diabetic rats did not improve the reduced transcriptional activity, although it is documented that direct addition of T_3 normalizes the decreased transcriptional activity in hypothyroid rats (5). These findings suggest that hypothyroidism did not affect the regulation of mitochondrial genes in diabetes.

Interestingly, the present findings indicated that protein synthesis in mitochondria of diabetic hearts was decreased. Previous studies showed that the content of cytochrome *b* in the liver of diabetic rats was decreased (20, 31); however, its mRNA content and mitochondrial protein synthesis in vitro were not investigated. In the present study, we found that not only decreased mRNA content of cytochrome *b* but also decreased capacity of mitochondrial protein synthesis contributed to the decreased content of cytochrome *b* in diabetes mellitus. Details of the mitochondrial protein synthesis are poorly understood, and the mechanism of decreased mitochondrial translation in the hearts of diabetic rats is unknown at present. However, these reduced levels of mitochondrial transcription and translation may present important pathophysiological significance in the hearts of diabetics. In the heart-specific mtTFA knockout mice, genes and protein expression of mitochondrial-encoded respiratory chain subunits are decreased, and these mice show the time-dependent deterioration of the respiratory chain function in the affected organs (17). From the findings of this previous study and of the present study, it is suggested that the reduced binding activity of mtTFA might contribute to the mitochondrial dysfunction in the heart of diabetic rats. Furthermore, additional generation of reactive oxygen species by the mitochondrial dysfunction might further decrease the binding activity of mtTFA. This idea implies that the reduced binding activity of mtTFA in diabetes might fall into a vicious cycle. In conclusion, the present study is the first to demonstrate that reduced mtTFA function impairs mitochondrial transcription in the hearts of diabetic rats. Although the amount of mtTFA protein in the mitochondrial matrix of diabetes was not changed, the binding activity of mtTFA to the D-loop region was decreased.

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