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The Role of Increased Oxidative Stress in the Development of Diabetic Nephropathy

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ABSTRACT

The pathogenesis of diabetic nephropathy is still not completely understood while renal disease is one of the most common disabling complications of diabetes. We, in the present study, investigated the possible involvement of oxidative stress in the development of diabetic nephropathy. To hasten the development of diabetic nephropathy, streptozotocin was injected to unilaterally nephrectomized rats (NEPH-STZ). Eight weeks later, NEPH-STZ rats developed severe hyperglycemia, proteinuria, and hypertension. The kidneys of these rats showed compensatory hypertrophy and mesangial expansion. In contrast, the rats with streptozotocin injection alone (STZ) did not increase urinary protein excretion. Nephrectomized non-diabetic rats (NEPH) developed increased urine protein excretion, but without prominent renal morphological changes. However, oxidation of renal cortical tissue protein significantly increased in all 3 groups of NEPH, STZ and NEPH-STZ in comparison to control rats (CONT). The result indicates the non-specificity of the oxidative tissue damage and suggests that the oxidative damage is hardly a sole mechanism leading to the development of the diabetic nephropathy. However, it would still be a contributing factor considering that the oxidative stress is a common final pathway mediating tissue damages in chronic diabetic complications and other serious illness.

Key Words: Oxidation, Diabetes, Nephropathy, Nephrectomy, Streptozotocin

INTRODUCTION

Renal disease is one of the most common disabling complications of diabetes mellitus. Diabetic nephropathy characteristically develops persistent proteinuria, hypertension, and progressive loss of renal function (Foster, 1991). It frequently leads to gradual renal structural damages terminating in end-stage renal disease. Although clinical and pathological characteristics of diabetic nephropathy are well defined, its pathogenesis is still incompletely understood.

Oxidative stress has been proposed as a common pathway linking diverse mechanisms for the pathogenesis of chronic complications in diabetes (Winegrad, 1987; Baynes, 1991). This hypothesis suggests the possibilities that the diabetic state lead to increases in reactive oxygen species production or to decreases in the clearance of or protection against them. Indeed, changes in scavenger systems such as superoxide dismutase (SOD; Matkovics *et al.*, 1982) and catalase(CAT; Bistar *et al.*, 1983) activities, glutathione (GSH) metabolism (El-Hawary *et al.*, 1977), and vitamin E concentra-

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tions (Karpen *et al.*, 1982) have been observed in diabetes. In addition, chemical modifications of proteins and lipids, which appear to be largely oxidative in origin, are associated with diabetes with complications (Baynes, 1991).

Various mechanisms may contribute to the increased oxidative stress in diabetes. These include increased non-enzymatic and autoxidative glycosylation, metabolic stress resulting from changes in energy metabolism, alterations in sorbitol pathway activity, changes in the level of inflammatory mediators and the status of antioxidant defense systems, and localized tissue damages resulting from hypoxia and ischemic reperfusion injury.

Although the increased oxidative stress is suggested to be involved in renal tissue damage in diabetes, there is no direct evidence of it. The purpose of the present study was to investigate the role of oxidative stress in the development of diabetic nephropathy. To accomplish this, oxidative damage of renal tissue proteins and lipids as well as functional and morphologic changes were measured in unilaterally nephrectomized diabetic rats.

MATERIALS AND METHODS

Materials

We purchased chemicals and assay kits from the following sources: streptozotocin, 1,1,3,3-tetraethoxypropane, thiobarbituric acid(TBA) hematoxylin, eosin, basic fuchsin, periodic acid, sodium metabisulfite, and activated charcoal (Sigma Chemical Co., St Louis, MO, USA); ethanolamine, acetonitrile, and cyclohexane (Aldrich Chemical Inc., Milwaukee, WI, USA); 2,3-dinitrophenylhydrazine (DNPH; Eastman Chemical Co, Rochester, NY, USA); Biorad protein Assay kit (Biorad Laboratories, Richmond, CA, USA); Glyc-Affin GHb kit (Iso Lab Inc, Akron, OH, USA); Diastix and Ketostix (Miles Sankyo Co, Tokyo, Japan)

Experimental animals

We utilized a combined approach of unilateral nephrectomy and streptozotocin injection to hasten the development of diabetic nephropathy (O' Donnel *et al.*, 1986; Anderson *et al.*, 1992). Six-week old male Sprague-Dawley rats were nephrectomized on the left side under pentobarbital anesthesia (45 mg/kg) and were injected with gentamycin of 2 mg i.p. daily for 3~5 days. Two weeks after the surgery, diabetes was induced by an intraperitoneal injection of streptozotocin (65 mg/kg). Only those rats with glucosuria of at least 2^+ were included for the study. The diabetic state was maintained for 8 weeks without insulin therapy. Urine glucose and ketone bodies were monitored twice a week with Diastix and Ketostix in these animals. Age matched control rats with either nephrectomy or streptozotocin injection alone, or without any of the two manipulations, were also included in the study. Eight weeks after the induction of diabetes, the rats were studied. The rats were anesthesized and a catheter was inserted into the portal vein. After collection of blood for glycated hemoglobin determination, the portal vein was perfused with ice-cold heparinized saline(1 U/ml) containing deferoxamine(1 μ g/ml) to remove blood from the kidneys for morphological and biochemical examinations.

Glycated hemoglobin (GHb) assay

GHb representing long-term hyperglycemia was measured with Glyc-Affin GHb kit using affinity chromatography (Abraham *et al.*, 1983).

Measurement of blood pressure and 24-hr total urine protein excretion

Arterial blood pressure and 24-hr total urine protein were measured before the nephrectomy and 1~3 days prior to the kidney perfusion in all experimental animals.

The blood pressure was measured in unanesthetized rats by the tail-cuff method (Pfeffer *et al.*, 1971) using a physiograph (IITC, Life Sciences) in a quiet environment. The average of 3 successive readings was reported.

Rats were individually housed in metabolic cages to collect a 24-hr urine sample. During urine collection, rats were deprived of food, but had free access to water. Urine protein concentration was measured with Bio-rad protein assay kit using globulin as a standard (Bradford, 1976) and 24-hr urine protein excretion was calculated using 24-hr urine volume.

Morphologic examinations

After the perfusion with heparinized saline, 2 midcoronal slices of 2 to 3 mm thickness were fixed in 10% formalin solution. Three micrometer thick paraffin sections were stained with periodic acid Schiff reaction (PAS; McManus, 1948). All tissue samples were examined under light microscope by an investigator without prior knowledge of the group to which the rat belonged.

The extent of increase in mesangial matrix was evaluated as previously dscribed(O'Donnell *et al.*, 1985). A semi-quantitative score was developed to evaluated the degree of glomerular damage. Thirty glomeruli were examined in each specimen and each glomerulus was evaluated by quadrants. Thus, a 1+ lesion representad 25% involvement of a glomerulus and a 4+lesion indicated that 100% of the glomerulus was involved. An injury score was obtained by multiplying the degree of involvement(0 to 4+) and the percentage of glomeruli with the same histologic change. The extent of injury in each individual tissue specimen was then assessed by the addition of this score. For example, if 5 among 30 glomeruli examined have an injury score of 1+ and 3 have a 2+ lesion, the final injury score in that specimen would be $((1\times3/30)+(2\times5/30)\times100=43$.

Measurement of tissue oxidation

The kidneys were excised following complete removal of RBC with the perfusion of saline. The renal cortical tissues separated from the medulla were freezed rapidly in liquid nitrogen. The freezed cortical tissues were powdered using mortar and pestle, and homogenized with a glass-teflon homogenizer.

In studies of mitochondrial membrane lipid peroxidation, mitochondrial suspensions were obtained by centrifugation of renal cortical homogenates in 0.25M sucrose solution at 600xg for a minute followed by centrifugation of the supernatant at 4800xg for 15 minutes for the final pellet (Paller *et al.*, 1984).

Protein oxidation was assessed by the determination of protein carbonyl group content using DNPH incorporation technique (Levine *et al.*, 1990; Jang *et al.*, 1993). Briefly, 2 equal aliquots of the supernatant fraction of cortical tissue homogenate were precipitated with trichloroacetic acid (TCA). One aliquat was reacted with 10 mM DNPH in 2N HCl for an hour in room temperature and the other one with 2N HCl only. The protein was precipitated again with TCA and the free DNPH was removed by washing with ethanol: ethylacetate (1 : 1, v/v). Finally, the protein was dissolved in 6M guanidine HCl solution. The difference spectrum of the DNPH-reacted vs. the HCl control was determined using spectrophotometer (Spectronic 601, Milton Roy Co., Oostende, Belgium), which exhibited a maximum absorbance at 360~370 nm. The concentration of carbonyl group was calculated from the absorbance at 365 nm using an average value of 21.0 $M^{-1}cm^{-1}$ for the molar absorptivity of aliphatic DNPH derivatives.

In order to measure the content of conjugated dienes (CD), the total lipid was extracted from cortical homogenates or mitochondria-enriched fraction with chloroform/methanol (1 : 1, v/v) and chloroform/methanol/water (86 : 14 : 1; Pryor *et al.*, 1984). The lipid extract was dried completely under pure nitrogen gas (99.999%) which passed through oxygen trap of heated cupper coil. The dried extract was redissolved in cyclohexane and its absorbance at 234 nm measured against a solvent blank by spectrophotometer. The concentration of CD was calculated using 2.52 ± 10^{-5} $M^{-1}cm^{-1}$ as the molar extinction coefficient of CD.

MDA content was measured with HPLC following TBA reaction (Therasse *et al.*, 1987). Cortical homogenate or mitochondria-enriched fraction in TCA was reacted with TBA reagent in 95°C water bath for 30 min. After extraction with ethyl acetate containing diethyl ether, the concentration of TBA-MDA complex was quantified at 592 nm absorbance using ODS c-18 column(μBondapak, waters) and 0.1% ethanolamine/acetonitrile (2 : 1, V/V) as a mobile phase.

The contents of MDA, CD, and protein carbonyl group were represented as nmoles per mg protein and measured in duplicate. Tissue protein was measured by a assay kit (Bradford, 1976).

Statistical analysis

Values are mean±standard error. Statistical analysis was performed with one-way analysis of variance at a significance level of $P<0.05$ using Stat View IV (Abacus Concepts, 1992) statistical

program. Further specificgroup differences were determined with Fisher's protected least significant difference test (PLSD).

RESULTS

Body weight (BW), glycated Hb (GHb), urine protein excretion, and blood pressure

Normal rats (CONT) gained 193±18g of BW during the 8 weeks of animal preparation period (Table 1). BW was significantly reduced in streptozotocin injected rats with (NEPH-STZ) or without (STZ) prior nephrectomy, demonstrating a severe emaciation in diabetes. Nephrectomized rats without streptozotocin injection (NEPH) gained as much as CONT rats.

Glycated Hb in NEPH and CONT was around 4% that was within normal range(Table 1). All the streptozotocin injected rats showed a marked increase in GHb, 15.8±0.4% in STZ and 16.5±0.4% in NEPH-STZ.

Urine protein excretion, shown in Fig. 1, increased significantly in NEPH-STZ (28.0±3.5 mg/24hr) rats in comparison to CONT rats (12.7±0.7 mg/24h). NEPH rats also showed high urine protein (21.4±1.9 mg/24h), which was in accordance with a previous report (Garcia *et al.*, 1990). Streptozotocin injection alone did not increase urine protein excretion.

Systolic and mean arterial pressure (MAP) of CONT group were 128±2 and 92±2 mmHg, respectively (Fig. 2). Blood pressure significantly increased in STZ rats. Hypertension was even more aggravated in NEPH-STZ group, resulting

Table 1. Body Weight Changes (△BW) during 8 weeks of experimental animal preparation and glycated hemoglobin (GHb) in control (CONT), nephrectomy (NEPH), streptozotocin(STZ), and nephrectomy+streptozotocin (NEPH-STZ) groups

EXP. Group	n	△BW(g)	GHb(%)
CONT	16	193±18	4.2±0.9
NEPH	13	155±15	4.2±0.1
STZ	14	−30±18*	15.8±0.4*
NEPH-STZ	40	49±10*	16.5±0.4*

P<0.05 vs. CONT

Table 2. Glomerular morphology in experimental animals

Group	Mesangial expansion score
NEPH	9.6± 5.2
STZ	37.8±10.8*
NEPH-STZ	35.6± 9.4*

P<0.005 vs. NEPH

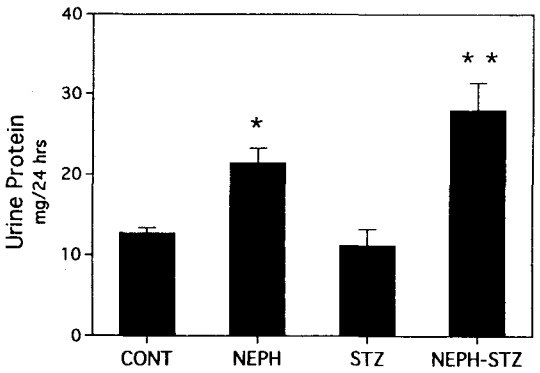

Fig. 1. Urine protein excretion(mg) during 24 hrs.
*P<0.05 vs. CONT,
**P<0.005 vs. CONT and STZ

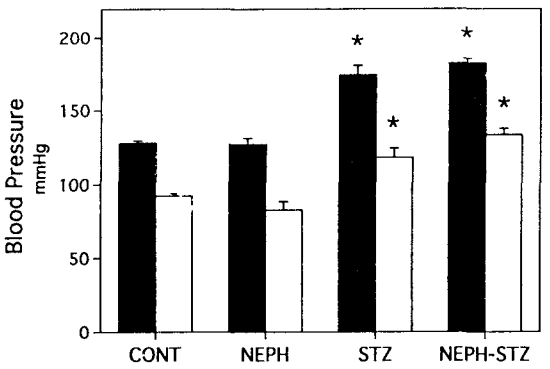

Fig. 2. Systolic(dark bar) and mean arterial blood pressure(light bar) in experimental animals.
* P<0.05vs. CONT

140% of CONT values in both of MAP and systolic pressure. Nephrectomy alone, however, did not affect blood pressure.

Morphologic changes

The glomerular morphologic changes are summarized in Tabel 2. Nephrectomized non-diabetic rats did not develop significant morphologic changes except hypertrophy (data not shown). In contrast, STZ and NEPH-STZ rats demonstratd a significant mesangical matrix expansion.

Protein and lipid oxidation

There was a significant difference in renal cortical tissue protein oxidation, represented as carbonyl group content, between groups. It increased from 1.44 ± 0.09 nmoles/mg protein in CONT to 2.05 ± 0.10, 1.89 ± 0.15, 2.17 ± 0.08 nmoles/mg protein in NEPH, STZ, NEPH-STZ groups, respectively (Fig. 3).

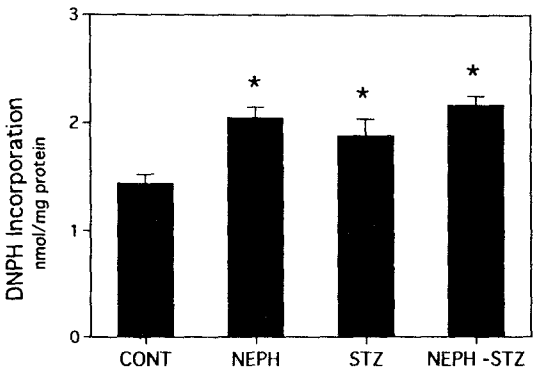

Fig. 3. Protein carbonyl group contents in renal cortical tissue represented by DNPH incorporation.
* $P<0.05$ vs. CONT

The contents of CD and MDA measured in renal cortical homogenates are essentially the same as that measured in mitochondria-enriched fraction ($P=0.8$ or worse). Therefore, we used both data for the comparison between groups. The content of CD was 5.07 ± 0.54 nmoles/mg protein in CONT group and did not change with nephrectomy, streptozotocin injection, or both (Fig. 4A). Changes in MDA contents are shown in Fig. 4B. The content of MDA was about 50% higher in NEPH-STZ than in CONT. However, the difference was not statistically significant due to large variance in MDA measurements.

DISCUSSION

The rats in NEPH-STZ group developed severe hyperglycemia, proteinuria, and hypertension (Table 1, Fig. 1 & 2). The remaining kidneys of these rats showed compensatory hypertrophy (data not shown) and mesangial expansion (Table 2). The animals of NEPH-STZ group thus successfully presented all of the characteristics of diabetic nephropathy. On the other hand, STZ rats showed increased arterial pressure and renal

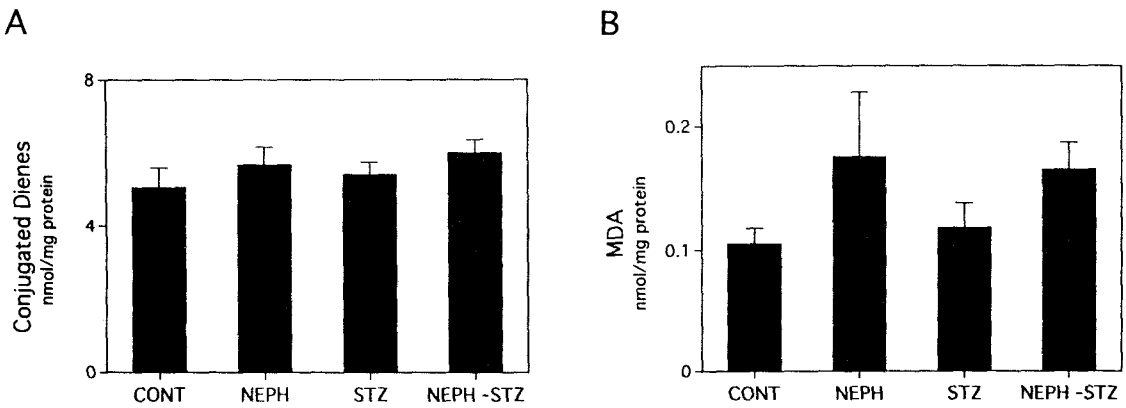

Fig. 4. The contents of conjugated dienes and malondialdehyde(MDA) in renal cortical tissues.

mesangical expansion without accompanying excessive protein excretion. Nephrectomized non-diabetic rats developed increased urine protein excretion, but without prominent renal morphological changes.

Oxidative tissue damage existed in NEPH, STZ, and NEPH-STZ rats as evidenced by the increased contents of protein carbonyl groups in renal cortical tissues (Fig. 3). Hence, tissue oxidative stress increased in all of 3 experimental group while morphological changes of diabetic nephropathy occurred only in diabetic rats. The result shows the non-specificity of the oxidative tissue damage, which increased in all three experimental conditions. These suggest that the oxidative damage is hardly a sole mechanism leading to the development of the diabetic nephropathy. However, it would still be a contributing factor, considering that the oxidative stress is a common final pathway mediating tissue damages in chronic diabetic complications and other serious illness (Weiss *et al.*, 1982; Croos *et al.*, 1987; Oberley, 1988; Shan, 1989; Baynes, 1991). We can conceptualize from this point that diabetic nephropathy is caused by a complex mechanism involving several pathogenic factors including hemodynamic (e. g. unilateral nephrectomy) and metabolic derangement (e.g. hyperglycemia and hypoinsulinemia), and each factor provokes renal tissue damage at least partly through increasing oxidative stress. The demonstration of the reversal of renal damage by anti-oxidant treatment in nephrectomized and/or streptozotocin injected rats will help to prove this hypothesis.

The results reporting the changes in renal antioxidant status of diabetic rats also indicates the possible involvement of oxidative stress in the diabetic renal damage. Renal SOD and CAT activities decreased and glutathione peroxidase increased in the streptozotocin-induced diabetic rats (Wohaied *et al.*, 1987). They also found decreased CAT and GSH in the kidneys of spontaneously diabetic BB Wistar rats (Wohaieb *et al.*, 1987).

The contents of oxidized protein in the renal cortical tissues were similar in all of three experimental groups of NEPH, STZ, NEPH-STZ while the extent of renal damage represented by functional and morphological parameters was quite different between groups. This suggests, as mentioned above, the complexity of the mechanism involving in the development of renal damage in diabetes. Moreover, there is a certain limit for the accumulation of oxidized products of proteins or lipids due to the increased turn-over, for damaged proteins and lipids are much more vulnerable to the enzymatic degradation (Strake-Reed *et al.*, 1989).

Increased lipid peroxidation was not observed in any of experimental animal groups (Fig. 4). This was true for both of renal cortical homogenates and mitochondria enriched fractions. The failure in the detection of lipid peroxidation does not deny the presence of oxidative stress. The reason why lipid peroxidation was not observable despite the presence of protein oxidation is not clear at this point. Probably, in this particular situation, either CD or MDA do not represent the increases of lipid peroxides because they are only the two among the several intermediate products with rapid turn-over rate involved in the lipid oxidation cascade (Gutteridge *et al.*, 1990).

In conclusion, the present study provides the evidence that increased oxidative stress contributes to the development of diabetic nephropathy even though it may not be the only or major factor. The result also suggests the possibilities that administration of antioxidants may reduce or prevent the progression of nephropathy in chronic diabetes.

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=국문초록=

당뇨병성 신증의 발생에 있어서 산화성 스트레스의 역활

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당뇨병성 신증은 만성 당뇨병에서 흔히 동반되는 심각한 합병증이나 그 발생기전은 확실히 밝혀져 있지 않다. 본 연구에서는 암, 노화, 염증등 여러가지 질환의 발생에 관여한다고 알려진 산화성 스트레스의 증가가 당뇨병성 신증의 발생에 관여하는지를 알아 보았다. 쥐에서 신증의 유발을 용이하게 하기 위해 먼저 한쪽 신장을 적출한후 스트렙토조토신을 주사하여 당뇨병을 유발하였다(NEPH-STZ). 당뇨병 유발 8주후, NEPH-STZ쥐들은 심한 고혈당증, 단백뇨, 고혈압의 증상을 보였으며 신장조직은 보상성 비대와 함께 혈관간기질(mesangial matrix) 증대의 형태학적 변화를 보였다. 그러나 스트렙토조토신만을 주사한 쥐(STZ)에서는 단백뇨가 나타나지 않았고, 신장적출만을 한 쥐에서는(NEPH) 단백뇨의 증상은 나타났으나 신장조직의 형태학적 관찰에서 혈관간기질의 증대는 보이지 않았다. 이와같이 그 증상이나 신장조직 병변의 정도는 모두 달랐음에도 불구하고, 신피질 조직 단백질의 산화는 NEPH, STZ, NEPH-STZ의 세 실험군 쥐에서 대조군(CONT)에 비해 유의하게 증가 되어 있었다. 이 실험결과는 세 실험군 모두에서 조직의 산화성 스트레스가 증가되어 있음을 나타내는 것으로, 산화성 스트레스의 증가가 당뇨병성 신증을 일으키는 유일한 발병기전은 아닐 가능성을 시사한다. 그러나 조직의 산화성 스트레스의 증가가 여러 질환에서 조직의 손상을 유발하는 공통된 최종 경로라는 것을 고려해 볼 때, 혈행학적(hemodynamic) 혹은 대사성 인자 등에 의해 증가된 산화성 스트레스가 당뇨병성 신증의 발생에 있어서도 기여할 가능성은 크다.