Endothelin-1 (ET-1) plays an important role in the pathogenic mechanism of diabetic nephropathy. However, the regulatory effects of ET-1 on superoxide and prostaglandin E2 (PGE2) in diabetic glomeruli are unclear. The aim of this study was to determine whether ET-1 exerts a differential effect on the production of superoxide and PGE2 in diabetic glomeruli. The regulatory effects of indomethacin, insulin, dexamethasone, and heparin were also investigated. Freshly isolated glomeruli were obtained from normal and streptozotocin-induced diabetic rats for 1 week (DM1W), 1 month (DM1M), and 3 months (DM3M), respectively. Our results showed that the basal superoxide production of isolated glomeruli was significantly higher in DM1M and DM3M than in the normal rats ($p<0.01$). ET-1 stimulated superoxide production in normal, DM1W and DM1M glomeruli ($p<0.01$) but not in DM3M rats. The basal production of PGE2 in isolated glomeruli did not differ between diabetic and normal rats. ET-1 also stimulated PGE2 production in diabetic rats ($p<0.05$). Pretreatment with indomethacin further enhanced ET-1-stimulated superoxide production in all groups of diabetic rats ($p<0.05$), while the ET-1-stimulated PGE2 production was attenuated by indomethacin. Insulin, dexamethasone and heparin had no additional effects on ET-1-mediated superoxide and PGE2 production. In conclusion, basal glomerular production of superoxide but not PGE2 was increased in the diabetic glomeruli. ET-1 further stimulated production of both superoxide and PGE2. Indomethacin could enhance ET-1-stimulated superoxide production while attenuating PGE2 production.

**Key Words:** diabetic rats, endothelin-1, isolated glomeruli, prostaglandin E2, superoxide

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide originally isolated and purified from the conditioned medium of cultured porcine aortic endothelial cells [1]. It has been shown to process a wide spectrum of biological activity in the kidneys [2]. ET-1 may play different roles in the diabetic kidney because the diabetic glomeruli exhibit a poor contractile response to vasoactive agents and diabetic kidneys do not contract, even during end-stage renal failure. The synthesis of ET-1 can be induced by various stimuli, including shear stress, hypoxia, cytokines, thrombin, angiotensin II and transforming growth factor-α [3]. All these stimuli can be found in renal glomeruli, the functional unit of the kidneys, and especially in the glomerular mesangial cells.

ET-1 induces the proliferation of mesangial cells and the production of extracellular matrix [4]. Cellular proliferation and increased extracellular matrix are major factors involved in glomerulosclerosis, playing...
important roles in the progression of renal diseases including diabetes [5]. We have recently demonstrated both the production of ET-1 and the expression of ET-1 mRNA is increased in the glomeruli of diabetic rats. Glomerular production of ET-1 can be enhanced by superoxide, one of the major reactive oxygen species (ROS). However, the effects of ET-1 on the production of superoxide and prostaglandin E2 (PGE2) in the whole glomerulus, the functional unit and the major target of diabetic insult, is unclear. This issue becomes important because ET-1 has been identified as an autocrine cytokine [6]. Therefore, the aim of this study was to determine whether ET-1 exerts a differential effect on the production of superoxide and PGE2 in the diabetic glomeruli. Both are thought to play an important role in the underlying pathophysiology of diabetic nephropathy. We also investigated the regulatory effects of indomethacin, insulin, dexamethasone, and heparin on ET-1-mediated production of superoxide and PGE2. All these agents have been described to exert different biological and pharmacological effects in patients with glomerular disorders. Among them, indomethacin, a non-steroid anti-inflammatory drug, is a prostaglandin inhibitor and also the cause of renal disorders. Insulin, the drug used for treating diabetes, is known to have anti-oxidative effects [7]. Dexamethasone, a corticosteroid, is the major immunosuppressant for treating immunological renal disorders. Heparin has been reported to regulate ET-1 and superoxide [7].

**Materials and Methods**

Male Wistar rats with a body weight between 200–250 g were used. Rats were injected with 55 mg/kg of streptozotocin (Sigma Chemical Co., St Louis, MO, USA) intraperitoneally and used in experiments after confirming the diagnosis of diabetes mellitus. Rats were classified into three groups, each containing 10 rats. The different groups represented different periods of diabetes. Group I was sacrificed at 1 week after induction of diabetes (DM1W), Group II was sacrificed at 1 month (DM1M) and Group III at 3 months (DM3M). All three groups had the same number of rats sacrificed at the same time to serve as normal controls. Group II and III were administered insulin (heat-treated bovine ultralente insulin; Novo-Nordisk, Copenhagen, Denmark) every day to maintain a poorly controlled diabetic state. Plasma glucose levels were regularly checked and maintained above 450 mg/dL. All rats were allowed food and water ad libitum. The animal experiment was approved by the animal committee of Kaohsiung Medical University.

**Experimental protocol**

Freshly isolated diabetic or normal rat glomeruli were stimulated with ET-1 at a concentration of 50 nM. The concentration was chosen after a dose-response study. The production of superoxide and PGE2 were measured at basal levels and after ET-1 stimulation for 30 minutes. The production of diabetic and normal glomeruli was conducted with or without indomethacin (1.0 μM), insulin (0.3 mU/mL), dexamethasone (0.1 μM), or heparin (100 μg/mL), and these were pre-incubated at 37°C for 2 hours before stimulation with ET-1. To test the effect of insulin, the diabetic or normal glomeruli were initially incubated with insulin at 4°C for 90 minutes, and then incubated for 30 minutes at 37°C. It should be emphasized that an initial exposure of diabetic glomeruli to insulin at low temperature was required for expression of its effects [8]. All chemicals were purchased from the Sigma (St Louis, USA) unless otherwise specified.

**Preparation of glomeruli**

Diabetic and normal rats were anesthetized with pentobarbital and then perfused by aorta puncture with 30 mL Hank’s solution and 10 mL RPMI 1640 medium [9]. After this, bilateral nephrectomy was performed, the renal capsule removed, and the renal cortex dissected and removed from the perfused kidneys. The glomeruli were obtained by pressing minced renal cortices through sieves of graded sizes. Using this method, we could obtain glomeruli at 95% purity. The viability of the isolated glomeruli was regularly checked with a lactic dehydrogenase assay. All glomeruli were shown to be viable at the end of each experiment. The collected glomeruli were then incubated on 24-well tissue culture plates at 1 × 10^4 glomeruli/mL in RPMI 1640 medium supplemented with 20% fetal calf serum and antibiotics. All glomeruli were used immediately after isolation and all experiments were finished within 6 hours. By the end of each experiment, glomeruli were homogenized and the protein content was measured using the Bradford assay (Bio-Rad, Hercules, CA, USA).
Measurement of superoxide
Superoxide anion production by glomeruli was determined by measuring the superoxide dismutase-inhibiting reduction of cytochrome C by a spectrophotometer [7]. Briefly, the glomeruli (containing $1 \times 10^4$ glomeruli/mL) in phosphate buffered saline containing $\text{Ca}^{2+}$ (1 mM) and cytochrome C (80 μM) with or without superoxide dismutase (500 μg/mL) were preincubated for 10 minutes in a 37°C water bath. The glomeruli were then stimulated with ET-1 at 37°C for 6 hours in a vibrating water bath and the reaction terminated by placing on ice. The absorbance of the supernatant was then measured at 550 nm.

Measurement of PGE2
PGE2 was extracted from the supernatant and measured using a radioimmunoassay kit (Amersham, Arlington Heights, IL, USA). The samples were purified after adding ethanol and glacial acetic acid and centrifuged. The supernatant was applied to a C18 Sep-Pak cartridge. After being washed with water and hexane, the samples were eluted with ethyl acetate and dried with nitrogen. The extracted samples were reconstituted with methyl oxidation reagents and analyzed by radioimmunoassay.

Statistical analysis
All results are expressed as mean ± standard error mean. Unpaired $t$ tests were used to compare differences.

RESULTS
Superoxide production
The basal production of superoxide in isolated normal glomeruli was $0.84 \pm 0.11$ nmol/mg glomerular protein/30 min, which was significantly lower than that in DM1M and DM3M rats ($p<0.01$ for both groups, Figure 1). The superoxide production was also significantly higher in the glomeruli isolated from DM3M rats than that isolated from DM1W rats ($p<0.01$). ET-1 enhanced superoxide production in normal, DM1W, and DM1M groups (all $p<0.01$), but not in the DM3M group. Pretreating the glomeruli with indomethacin enhanced superoxide production in all diabetic groups (all $p<0.05$) but not in the normal controls, while insulin, dexamethasone and heparin did not have an effect in any of the groups (Table 1).

Table 1. Superoxide production in freshly isolated glomeruli after stimulating with endothelin-1 for 30 minutes with or without indomethacin, insulin, dexamethasone and heparin in normal and streptozotocin-induced diabetic rats*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal</th>
<th>DM1W</th>
<th>DM1M</th>
<th>DM3M</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 only</td>
<td>$5.12 \pm 0.87$</td>
<td>$3.97 \pm 0.28$</td>
<td>$3.16 \pm 0.21$</td>
<td>$3.08 \pm 0.20$</td>
</tr>
<tr>
<td>ET-1 + indomethacin</td>
<td>$4.99 \pm 0.21$</td>
<td>$4.96 \pm 0.32^*$</td>
<td>$3.87 \pm 0.20^*$</td>
<td>$3.82 \pm 0.19^*$</td>
</tr>
<tr>
<td>ET-1 + insulin</td>
<td>$5.01 \pm 0.38$</td>
<td>$4.09 \pm 0.29$</td>
<td>$3.22 \pm 0.30$</td>
<td>$3.12 \pm 0.19$</td>
</tr>
<tr>
<td>ET-1 + dexamethasone</td>
<td>$5.33 \pm 0.39$</td>
<td>$3.88 \pm 0.21$</td>
<td>$3.09 \pm 0.27$</td>
<td>$3.28 \pm 0.18$</td>
</tr>
<tr>
<td>ET-1 + heparin</td>
<td>$5.20 \pm 0.47$</td>
<td>$3.69 \pm 0.37$</td>
<td>$3.19 \pm 0.27$</td>
<td>$3.01 \pm 0.21$</td>
</tr>
</tbody>
</table>

*Data presented as mean ± standard error mean of triplicate samples from six independent experiments; $^p<0.01$ compared with ET-1 group. ET-1 = endothelin-1; DM1W = diabetic rats for 1 week; DM1M = diabetic rats for 1 month; DM3M = diabetic rats for 3 months.

Figure 1. Superoxide production of freshly isolated rat glomeruli stimulated with or without endothelin-1 treatment (50 nM) for 30 minutes in normal and streptozotocin-induced diabetic rats. Data are mean ± standard error mean of triplicate samples from six independent experiments. $^*p<0.01$ for comparison between rats with and without ET-1 at the same time point; $^†p<0.01$ for comparison of rats without ET-1 to normal; $^‡p<0.01$ for comparison of rats without ET-1 to DM1W. ET-1 = Endothelin-1; DM1W = diabetic rats for 1 week; DM1M = diabetic rats for 1 month; DM3M = diabetic rats for 3 months.
production did not significantly differ between any of the diabetic groups. ET-1 enhanced PGE2 production in all the diabetic groups (all \( p < 0.05 \)) but not in the normal controls. Pretreating the glomeruli with indomethacin attenuated ET-1-enhanced PGE2 production in normal and all diabetic groups (\( p < 0.05 \)) but not in the normal controls. Pretreating the glomeruli with indomethacin attenuated ET-1-enhanced PGE2 production in normal and all diabetic groups (\( p < 0.05 \)) but not in the normal controls. Pretreating the glomeruli with indomethacin attenuated ET-1-enhanced PGE2 production in normal and all diabetic groups (\( p < 0.05 \)) but not in the normal controls.

**DISCUSSION**

In this study, we demonstrated that basal production of superoxide but not PGE2 was increased in freshly isolated diabetic glomeruli. ET-1 further enhanced production of both superoxide and PGE2 in diabetic glomeruli. The kidney is an important organ for the synthesis of ET-1. We previously demonstrated basal ET-1 production of diabetic glomeruli increased with the progression of diabetes [10], and insulin added in vitro partially attenuated ET-1 production by diabetic glomeruli. The major cause of increased basal ET-1 production by diabetic glomeruli may be hyperglycemia, as we found that high glucose concentration in vitro enhanced ET-1 production in normal glomeruli [10]. There have been several studies demonstrating that ET-1 receptors are widespread in the kidneys [11,12]. Overexpression of endothelin receptors can be found in the renal cortex of diabetic rats [12], and endothelin receptor antagonists can prevent the progression of diabetic nephropathy [13,14]. Therefore, the effects of ET-1 on isolated glomeruli can be expected.

Freshly isolated glomeruli are the best materials for studying living tissues, since nearly all the glomeruli have been shown to be viable by the end of the study. Basal production of cytokines or hormones under this condition mimics the in vivo status, and reflects the results of various interactions common in the glomerulus. We have demonstrated that basal production of superoxide is increased in isolated diabetic glomeruli compared with normal controls, and production increased further with the progression of diabetes. Superoxide is the major ROS which has been implicated in the pathogenesis of diabetic nephropathy [10]. Glomeruli have been shown to generate ROS [15], and there is considerable evidence suggesting generation of ROS increases in diabetes [16,17] and in acute hyperglycemia [18]. During diabetes, persistent hyperglycemia causes increased production of ROS through autooxidation of glucose and by non-enzymatic protein glycation, which may not only disrupt cellular functions [17] but also inactivate the endogenous ROS scavengers including superoxide dismutase and catalase [19], causing an endogenous

**Table 2.** Prostaglandin E2 production in freshly isolated glomeruli after stimulating with endothelin-1 with or without indomethacin, insulin, dexamethasone and heparin in normal and streptozotocin-induced diabetic rats normal and diabetic glomeruli*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal</th>
<th>DM1W</th>
<th>DM1M</th>
<th>DM3M</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 only</td>
<td>1.48±0.37</td>
<td>2.58±0.47</td>
<td>3.12±0.56</td>
<td>3.34±0.64</td>
</tr>
<tr>
<td>ET-1 + indomethacin</td>
<td>0.32±0.19</td>
<td>0.86±0.47†</td>
<td>0.69±0.30‡</td>
<td>1.04±0.28‡</td>
</tr>
<tr>
<td>ET-1 + insulin</td>
<td>1.26±0.52</td>
<td>2.62±0.98</td>
<td>3.42±1.08</td>
<td>3.15±0.88</td>
</tr>
<tr>
<td>ET-1 + dexamethasone</td>
<td>1.82±0.69</td>
<td>2.23±0.84</td>
<td>2.89±1.19</td>
<td>2.66±0.64</td>
</tr>
<tr>
<td>ET-1 + heparin</td>
<td>1.32±0.86</td>
<td>2.78±0.69</td>
<td>2.97±0.76</td>
<td>2.87±0.54</td>
</tr>
</tbody>
</table>

* Data presented as mean±standard error mean of triplicate samples from six independent experiments; † \( p < 0.05 \) compared to ET-1 group; ‡ \( p < 0.005 \) compared to ET-1 group. ET-1 = endothelin-1; DM1W = diabetic rats for 1 week; DM1M = diabetic rats for 1 month; DM3M = diabetic rats for 3 months.
oxidant/antioxidant imbalance. Superoxide is one of the major ROS that may be stimulated by high glucose. The mechanisms by which hyperglycemia enhances superoxide production may be through activating the protein kinase C (PKC) system. The PKC of glomeruli from streptozotocin-induced diabetic rats have been reported to be activated within 1 week after induction of diabetes [20]. The exposure of normal isolated glomeruli to a high ambient concentration of glucose also activates PKC [20], and PKC has been shown to mediate the generation and release of superoxide [21].

We have also demonstrated that ET-1 enhanced glomerular production of superoxide in normal, DM1W, and DM1M rats, respectively. However, the ET-1-enhanced superoxide production decreased progressively with the duration of diabetes, and it was not significantly different after ET-1 stimulation in the DM3M group. The results indicate that longer duration of diabetes is associated with a refractory response to ET-1 upon ROS generation.

We also found that basal PGE2 production was not increased in diabetic glomeruli, while it was increased significantly after ET-1 stimulation. Basal production of PGE2 by isolated diabetic glomeruli has been reported to be either higher [22,23] or unchanged [24] compared with normal glomeruli. However, their response to ET-1 has not been reported. ET-1 stimulates vascular smooth muscles and mesangial cells to release PGE2, which can attenuate the vasoconstrictor and mitogenic effects of this peptide. In glomerular mesangial cells, PGE2 may be an important negative feedback modulator of ET-1-stimulated cellular contraction. ET-1 markedly increased PGE2 release by rat mesangial cells for at least 6 hours [25]. Inhibition of prostaglandin synthesis with non-steroid anti-inflammatory drugs enhances the constrictor actions of Angiotensin II and ET-1 on the vasculature of the kidney and on the glomerulus. The enhanced production of prostaglandins, in response to constrictor peptides, is both short-term and long-term. Therefore, ET-1 stimulation over the short-term and the long-term activates a modulatory feedback pathway that depends on upregulation of arachidonic acid release through phospholipase A2 and enhanced synthesis of prostaglandin [26]. These abnormalities in prostaglandin biosynthesis by diabetic glomeruli may contribute to the altered glomerular hemodynamics in this pathophysiologic setting. Another study showed that ET-1 evokes PGE2 production in mesangial cells through activating phospholipase A2 [27]. Increased PGE2 production has been reported in diabetic placenta compared with controls, but these levels are not modulated by ET-1 [28]. ET-1 concentrations were found to be increased in embryos from streptozotocin diabetic rats compared with controls. However, addition of ET-1 or bosentan (an endothelin A and endothelin B receptor antagonist) did not alter PGE2 generation in embryos from either the control or streptozotocin diabetic rats [29].

ET-1 is an autocrine peptide and therefore the effects of ET-1 on diabetic glomeruli in vivo are unavoidable. Since many drugs have been proposed to be effective in treating glomerular disorders, we have observed the protective effects of four of them on isolated glomeruli. We found indomethacin further enhanced ET-1-enhanced superoxide production. Superoxide not only induces lipid peroxidation but also enhances the formation of oxidized-low density lipoproteins with the consequence of further glomerular injury. Therefore, indomethacin may further worsen renal function in diabetic nephropathy. Insulin, dexamethasone and heparin did not have any additional effects on ET-1-mediated superoxide production. In contrast, indomethacin suppressed ET-1-enhanced PGE2 production of normal and diabetic glomeruli, further emphasizing its deleterious effects on diabetic kidneys. Although dexamethasone has been reported to decrease ET-1-stimulated PGE2 release in glomerular mesangial cells [25], we did not find similar effects on the isolated glomeruli. Therefore, the results of studies on glomerular cells may not be directly applied to functional glomeruli.

In conclusion, our results suggest the basal production of superoxide was enhanced, while that of PGE2 was unchanged in freshly isolated diabetic glomeruli, and ET-1 further enhanced the production of both superoxide and PGE2. Indomethacin enhanced ET-1-mediated superoxide production while attenuating PGE2 production, and can apparently worsen diabetic nephropathy. There are no additional effects of insulin, dexamethasone and heparin on ET-1-mediated production of superoxide and PGE2.

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內皮素 -1 誘發游離糖尿病腎絲球之過氧化物及前列腺素 E2 製造增加

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† 高雄醫學大學附設醫院腎臟內科
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內皮素 -1（以下簡稱 ET-1）作用於糖尿病腎臟，也許有其重要的角色。因為糖尿病腎絲球收縮反應較差，而且就算已經末期腎病，糖尿病腎臟也很少萎縮，本研究之目的即是探討 ET-1 是否直接對糖尿病腎絲球作用。本實驗將糖尿病大鼠的腎絲球，自以 Streptozotocin 誘發糖尿病後 1 週（以下簡稱 DM1W），1 個月（以下簡稱 DM1M），及 3 個月（以下簡稱 DM3M）時取出，研究觀察 ET-1 對腎絲球產生過氧化物及前列腺素 E2（以下簡稱 PGE2）的影響。結果發現，大鼠游離腎絲球之過氧化物的基礎產量，在 DM1M，DM3M 比正常對照組高 (p < 0.01)，加入 ET-1 會增強正常，DM1W 及 DM1M 大鼠腎絲球的過氧化物產量 (p < 0.01)，但 DM3M 無此現象。大鼠腎絲球 PGE2 的基礎產量，在糖尿病組和正常對照組並無差別，ET-1 則會增強各組糖尿病大鼠的腎絲球 PGE2 產量 (all p < 0.05)。以 Indomethacin 前處理，會增強 ET-1 刺激糖尿病組產生過氧化物的作用，但會減弱 ET-1 增強 PGE2 產生的作用。另外 Insulin，Dexamethasone，及 Heparin 之前處理則無影響。本研究結論，糖尿病大鼠腎絲球的基礎過氧化物的產量較高，ET-1 刺激會增強其過氧化物及 PGE2 的產生；加入 Indomethacin 則會增強 ET-1 所造成的過氧化物產量增加，但會減弱 PGE2 的產生。

關鍵詞：糖尿病大鼠，內皮素 -1，游離腎絲球，前列腺素 E2，過氧化物
(高雄醫誌 2010;26:350–6)