Fluvastatin Attenuates Diabetes-Induced Cardiac Sympathetic Neuropathy in Association With a Decrease in Oxidative Stress

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**Background:** Increased oxidative stress might contribute to diabetic (DM) neuropathy, so the effects of long-term treatment with fluvastatin (FL) on myocardial oxidative stress and cardiac sympathetic neural function were investigated in diabetic rats.

**Methods and Results:** FL (10 mg·kg⁻¹·day⁻¹, DM-FL) or vehicle (DM-VE) was orally administered for 2 weeks to streptozotocin-induced DM rats. Cardiac oxidative stress was determined by myocardial 8-iso-prostaglandin F₂α (PGF₂α) and NADPH oxidase subunit p22phox mRNA expression. Sympathetic neural function was quantified by autoradiography using ¹²⁵I- and ¹³¹I-metaiodobenzylguanidine (MIBG). FL did not affect plasma glucose levels but remarkably decreased PGF₂α levels compared with DM-VE rats (13.8±9.2 vs 175.0±93.9 ng/g tissue), although PGF₂α levels were below the detection limit in non-DM rats. FL significantly reduced myocardial P22phox mRNA expression. Cardiac ¹³¹I-MIBG uptake was lower in DM-VE rats than in non-DM rats, but the decrease was attenuated in DM-FL rats (1.31±0.08, 1.88±0.22, and 1.58±0.18 %kg dose/g, respectively, P<0.01). Cardiac MIBG clearance was not affected by the induction of DM or by FL, indicating that the reduced MIBG uptake in DM rats might result from impaired neural function.

**Conclusions:** FL ameliorates cardiac sympathetic neural dysfunction in DM rats in association with attenuation of increased myocardial oxidative stress. (Circ J 2010; 74: 468–475)

**Key Words:** Metaiodobenzylguanidine; Prostaglandin F₂α; Radioisotope; Statins

A utonomic neuropathy is a major complication of diabetes mellitus (DM) and is associated with high morbidity and mortality. Cardiac sympathetic neural dysfunction in diabetic patients can be identified using ¹²⁵I-metaiodobenzylguanidine (MIBG) or ¹¹C-hydroxyephedrine as radiotracers. The etiology of diabetes-induced cardiac sympathetic neural dysfunction remains unclear, but increased oxidative stress might be involved in its pathogenesis. Hyperglycemia induces oxidative stress via increased activity of the polyol pathway, nonenzymatic glucose oxidation yielding advanced glycation end-products, protein kinase C activation and superoxide overproduction via the mitochondrial electron transfer chain. However, whether diabetic neuropathy of the heart is associated with increased cardiac oxidative stress and whether a reduction in cardiac oxidative stress could attenuate neural dysfunction remain to be elucidated.

Three-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, might favorably affect mortality and morbidity among diabetic patients, independently of their cholesterol-lowering effects, attributable to a reduction of oxidative stress and improving endothelial dysfunction. Experimental animal and clinical studies have revealed a favorable effect of statins on diabetic peripheral neuropathy. However, because it is still unclear whether or not statins improve cardiac sympathetic neural dysfunction in DM, we investigated whether fluvastatin, which has a powerful antioxidative effect, could attenuate cardiac sympathetic neural dysfunction in DM and whether fluvastatin-induced attenuation of diabetic neural dysfunction is associated with a reduction in cardiac oxidative stress.

**Methods**

This study proceeded in accordance with the guidelines for animal experiments at the University of Toyama.
Diabetes-Induced Cardiac Neuropathy

**Table 1.** Body and Heart Weights and Hemodynamic Variables

<table>
<thead>
<tr>
<th></th>
<th>Non-DM</th>
<th>DM-VE</th>
<th>DM-FL</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>329±21</td>
<td>265±36**</td>
<td>252±33**</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.76±0.04</td>
<td>0.62±0.09*</td>
<td>0.61±0.08**</td>
</tr>
<tr>
<td>Heart/body weight ratio (×10³)</td>
<td>2.31±0.10</td>
<td>2.36±0.15</td>
<td>2.42±0.11</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>421±23</td>
<td>353±28**</td>
<td>380±50</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>118±17</td>
<td>127±10</td>
<td>122±13</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 vs Non-DM.

**Table 2.** Blood Glucose and Plasma Lipid Levels

<table>
<thead>
<tr>
<th></th>
<th>Non-DM</th>
<th>DM-VE</th>
<th>DM-FL</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>140±22</td>
<td>441±56**</td>
<td>410±109**</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>64±15</td>
<td>70±8</td>
<td>75±13</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>14±7</td>
<td>75±58*</td>
<td>77±27*</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 vs Non-DM.

TC, total cholesterol. Other abbreviations see in Table 1.

**Experimental Animals**

DM was induced in male Wistar rats weighing 250–300 g by an intraperitoneal injection of 65 mg/kg of streptozotocin (STZ, n=40). Non-DM control rats (n=14) were not injected with STZ. The rats with glucose levels >250 mg/dl at 1 week after STZ injection were considered diabetic (n=28) and used in the experiments. Two weeks later, fluvastatin (10 mg·kg⁻¹·day⁻¹, n=14) or vehicle (DM controls: 0.1% carboxymethyl cellulose, n=14) was orally administered by gavage for 2 weeks. Standard rat chow and tap water were provided ad libitum throughout the study.

Systolic blood pressure and heart rate were measured using an indirect tail-cuff method (BP-98A, Softron) and lipid peroxides (LPO) in plasma were determined using a hemoglobin–methylene blue method that selectively detects the absolute quantity of LOOH. Cardiac oxidative stress was assessed as the levels of 8-isoprostaglandin F₂α (PGF₂α) and nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase subunit p22α mRNA expression, and cardiac sympathetic neural function was assessed using 131I- and 125I-labeled MIBG.

**Radioactive MIBG Tracers**

FUJIFILM RI Pharma Co Ltd (Tokyo, Japan) prepared and supplied 131I- and 125I-labeled MIBG at a radiochemical purity >98%, and specific activity of 30–70 GBq/mmol.

**Cardiac MIBG Accumulation**

Dual-tracer autoradiography proceeded as described.²²,²³ Briefly, 0.37 MBq of 125I-MIBG was injected via the external jugular vein under pentobarbital sodium anesthesia (30 mg/kg, ip). Two hours later, 1.85 MBq of 131I-MIBG was intravenously injected and 30 min thereafter, the heart was removed and washed in cold saline. Specimens were frozen in isopentane, cooled in dry ice, embedded in methyl cellulose and cut into serial 20-μm thick transverse sections. The uptake of 131I-MIBG was initially determined by autoradiographic exposure on imaging plates (BAS-UR, Fuji Film, Japan) for 6h. We allowed the 131I-MIBG to decay for 75 days and then determined 125I-MIBG uptake after autoradiographic exposure for 30 days. A preliminary study showed that cross-talk between the 2 tracers was negligible (<2%) at the applied doses of 131I and 125I.

We analyzed the autoradiographic images to determine cardiac MIBG uptake and distribution using a computer-assisted imaging-processing system (BAS3000, Fuji Film), as described.²² Transverse sections of the left ventricle (LV) were divided into 4 segments at the level of the papillary muscles. Myocardial tracer accumulation in each region was normalized as a ratio (%) of the administered dose per gram of heart tissue (%kg dose/g), using graded 131I- and 125I-labeled standards. The washout rates (WR) of MIBG in whole hearts and in individual segments were calculated as follows:

\[
WR (%) = \frac{(131I\ accumulation - 125I\ accumulation) \times 100}{131I\ accumulation}
\]

**Cardiac PGF₂α Levels**

LV tissues were disrupted in a Polytron homogenizer with 50 mmol/L HCl and the homogenate was centrifuged for 5 min. The supernatant was extracted with ethyl acetate by centrifugation of 3,000 g for 5 min and the resulting organic layer was evaporated under a stream of nitrogen. The residue was dissolved in a mixture (50 μl) of acetonitrile and ethanol, and then 1 mmol/L HCl (2.5 ml) was added. Tissue extract was applied to Empore disk cartridges preconditioned with methanol and 1 mmol/L HCl. The cartridges were washed with 1 mmol/L HCl followed by heptane. We eluted PGF₂α with ethyl acetate containing 1% methanol, and evaporated the eluate under a stream of nitrogen. The residue was dissolved in 0.05% formic acid and acetonitrile, and the PGL₂ level was determined by high-performance liquid chromatography–electrospray ionization–mass spectrometry (API 4000™ LC/MS/MS, Applied Biosystems, Foster City, USA).²⁴ The detection limit of PGL₂ was 0.1 ng/g tissue.

**NADPH Oxidase Subunit p22α mRNA Expression**

NADPH oxidase subunit p22α mRNA expression in the myocardium was evaluated by real-time reverse transcription–polymerase chain reaction (PCR). Total RNA was ex-
**Figure 1.** (Left & Middle) Plasma levels of lipid peroxides (LPO) and myocardial 8-iso-prostaglandin F$_2$α (PGF$_2$α) in non-diabetic rats (non-DM, n=7 and 5, respectively), diabetic rats given vehicle (DM-VE, n=8 and 5, respectively), and diabetic rats treated with fluvastatin (DM-FL, n=7 and 5, respectively). (Right) Myocardial NADPH oxidase subunit p22$^{phox}$ mRNA expression in DM-VE (n=6) and DM-FL (n=6) rats. Level of PGF$_2$α is below detection limit in all non-DM and in 3 of 5 DM-FL rats. Means±SD.

**Figure 2.** Cardiac accumulation of $^{131}$I-MIBG and $^{125}$I-MIBG in non-diabetic rats (non-DM, n=7), diabetic rats given vehicle (DM-VE, n=6), and diabetic rats treated with fluvastatin (DM-FL, n=7). See text for details. Means±SD.
Figure 3. Examples of cardiac $^{131}$I-metaiodobenzylguanidine (MIBG) accumulation of non-diabetic (Non-DM), vehicle-treated (DM-VE) and fluvastatin-treated diabetic (DM-FL) rats and schematic illustration of left ventricle (LV) (Right lower panel). MIBG accumulation is relatively homogeneous within LV in each rat. Schematic illustration shows LV divided into anterior (Ant), lateral (Lat), inferior (Inf) and septal (Sep) wall segments to evaluate regional MIBG accumulation.

Figure 4. Cardiac regional accumulation of $^{131}$I- and $^{125}$I-metaiodobenzylguanidine (MIBG). *P<0.05 and **P<0.01 vs non-diabetic rats (Non-DM), #P<0.05 and ##P<0.01 vs diabetic rats given vehicle (DM-VE). DM-FL, diabetic rats treated with fluvastatin. Means ± SD.
tracted from 100 mg of LV tissue using Isogen (Nippon Gene, Japan), followed by digestion with DNase (Takara, Japan) to eliminate any contamination by genomic DNA. Total RNA samples were reverse transcribed with oligo (dT) primers by use of an RNA PCR kit (Takara ver 3.0, Japan). Quantitative real-time PCR analysis was performed with a sequence detector (Mx3000P, Stratagene) in a total volume of 20 μl containing 1 μl of cDNA, 10 μl of reagent (Brilliant II Fast QPCR Master Mix, Stratagene), 8 μl of diethylpyrocarbonate-treated water, and 1 μl of primer and probe sets (Applied Biosystems) specific for cDNAs encoding NADPH subunits p22phox and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR cycling programs were as follows: denaturation at 95°C for 1 min, primer annealing at 40°C for 5 s, and extension at 60°C for 20 s for 40 cycles. Gene expression of p22phox was normalized to that of GAPDH.

Statistical Analysis
Results are expressed as means±SD. Variables between 2 groups were compared using an unpaired t-test. Differences between groups were tested using a 1-way analysis of variance, followed by the Bonferroni test for multiple comparisons. A value of P<0.05 was considered statistically significant.

Results
Both the body and the heart weighed less in rats with STZ-induced DM than in the non-DM controls, but the ratio of heart to body weight did not differ between the 2 groups. Fluvastatin did not affect body and heart weights or the elevated blood glucose levels in the diabetic rats (Tables 1, 2).

Oxidative Stress
Plasma levels of LPO tended to be higher in the DM rats than in non-DM controls, although the difference did not reach statistical significance. Fluvastatin significantly decreased LPO levels (Figure 1). Cardiac levels of PGF2α were below the detection limit (<0.1 ng/g LV tissue) in the hearts of non-DM rats, but remarkably increased in all rats with STZ-induced diabetes (Figure 1). Fluvastatin for 2 weeks obviously decreased the PGF2α levels, which fell below the detection limit in 3 of 5 of the treated rats. Levels of NADPH oxidase subunit p22phox mRNA expression were significantly reduced by fluvastatin (Figure 1).

Cardiac MIBG Accumulation
The accumulation of both 131I- and 125I-MIBG in the LV was reduced in the DM rats compared with the non-DM group (Figure 2) and fluvastatin attenuated these decreases in the DM rats. Each LV was divided into 4 segments with regions of interest positioned on the LV anterior, septal, lateral and inferior walls at the level of the papillary muscle (Figure 3). Regional MIBG accumulation did not significantly differ in the non-DM rats, but the septal accumulation of 131I-MIBG and the septal and inferior accumulation of 125I-MIBG tended to be lower than in the corresponding anterior or lateral regions (Figure 4). The uptake of MIBG was significantly
reduced in all segments of DM rat hearts. Fluvastatin re-
stored the DM-induced reduction of both $^{131}$I- and $^{125}$I-MIBG accumulation in these segments, but the increase in $^{131}$I-MIBG accumulation reached statistical significance only in the lateral and inferior regions.

Neither the induction of DM nor fluvastatin affected the WR of MIBG from the LV, and regional differences in WR among the non-DM, DM or fluvastatin-treated DM rats did not reach significance (Figure 5).

**Discussion**

In the present study, cardiac oxidative stress was markedly increased in rats with STZ-induced DM and fluvastatin treatment for 2 weeks significantly reduced the myocardial levels of PGF$_{2\alpha}$ and NADPH oxidase subunit p22$^{\text{phox}}$ mRNA expression. Fluvastatin also attenuated the decreased cardiac MIBG uptake in the DM rats. Because neither the induction of DM nor fluvastatin treatment affected the WR of MIBG from the LV, the reduced MIBG accumulation in the DM rats might have resulted from impaired neural norepinephrine uptake rather than from enhanced sympathetic activation. In our study, diabetic sympathetic neural dysfunction was homogeneous within the LV, but 2 studies identified a more obvious MIBG reduction in the inferior region of the diabetic heart. Fluvastatin-induced recovery of neural dysfunction was also homogeneous in the present study.

**Effects of Fluvastatin on Cardiac Oxidative Stress**

The F2 isoprostanes comprise a family of prostaglandin F$_{2\alpha}$ isomers that are formed in situ from the fatty acid backbone that is esterified in membrane phospholipids. In many previous studies, PGF$_{2\alpha}$, a major F2 isoprostane, has been used as a reliable marker of oxidative stress in various tissues. Others have shown that oxidative stress is increased in both type I and type II DM. Hyperglycemia induces oxidative stress through several pathways, including enhanced aldose reductase activity, increased advanced glycation end-products, altered protein kinase C activity and mitochondrial overproduction of superoxide. Matsushima et al found that glutathione peroxidase overexpression improved LV diastolic function, accompanied by the attenuation of myocyte hypertrophy, interstitial fibrosis and apoptosis, in mice with STZ-induced DM. Similarly, the attenuation of oxidative stress by overexpression of the antioxidant protein, metallothionein, or treatment with antioxidant N-acetyl-cysteine improved myocyte function or fibrosis in the diabetic heart, and the increased urinary PGF$_{2\alpha}$ levels seen in diabetic patients can be reduced by improving their metabolic control.

The present study found that cardiac levels of PGF$_{2\alpha}$ in non-DM rats were below detection limits, but these levels increased in the DM rats, together with increased plasma levels of LPO, although the latter did not reach statistical significance. Long-term fluvastatin administration decreased both cardiac PGF$_{2\alpha}$ and plasma LPO levels to those found in the non-DM rats. The fluvastatin-induced reduction of myocardial oxidative stress was also confirmed by NADPH oxidase subunit p22$^{\text{phox}}$ mRNA expression in the myocardium. Statins decrease free radical generation in the vascular wall and myocardium, and fluvastatin exerts potent antioxidant activities as a free radical scavenger, a property that is derived from its unique chemical structure. Thus, the present findings show that fluvastatin reduces cardiac oxidative stress in DM rats.

**Cardiac MIBG Accumulation**

Impaired cardiac norepinephrine uptake in diabetic hearts determined using MIBG or $^{14}$C-hydroxyephedrine is accompanied by decreases in cardiac norepinephrine transporter density and nerve growth factor protein. The etiology of cardiac sympathetic nerve dysfunction in DM remains unclear, but the following mechanisms have been proposed: (1) degeneration of proteins critical to neural function by non-enzymatic glycosylation; (2) reduction of neurotrophic factors; (3) altered neural polyol metabolism; and (4) microvascular disease with impaired blood flow. However, increased oxidative stress in DM might be a major mechanism in the development of neuropathy. Oxidative stress can mediate the apoptosis of neurons and cause vascular impairment that leads to endoneural hypoxia and impaired neural function. Antioxidants, such as α-lipoic acid, acetylcarnitine and vitamins E and C, can ameliorate the nerve function deficits in diabetic neuropathy.

We found that 2-week treatment with fluvastatin attenuated the DM-induced reduction of cardiac MIBG accumulation in association with a reduction in cardiac oxidative stress. However, some investigators have reported increased cardiac norepinephrine uptake and increased cardiac sympathetic nerve activity in rats with STZ-induced DM. An increase in cardiac sympathetic activity accelerates MIBG turnover at nerve terminals, resulting in decreased cardiac MIBG accumulation in delayed images. However, the present study found the same cardiac MIBG WR in the rats, regardless of fluvastatin administration. These findings suggest that the fluvastatin-induced improvement in cardiac MIBG accumulation did not result from suppressed sympathetic nerve activity, but rather from an improvement in neural norepinephrine uptake. Ii et al demonstrated that rosvastatin improves sciatic nerve function in mice with type II DM via restored microcirculation and direct neurotrophic effects. Cameron et al reported that rosuvastatin improves sciatic and saphenous nerve conduction velocity in rats with STZ-induced DM. The present results suggest that improvement in diabetic sympathetic neuropathy induced by fluvastatin resulted from either suppressed neural oxidative stress or from an increase in endoneural blood flow through amelioration of oxidative stress-induced microvascular dysfunction in the diabetic heart.

Gill et al showed using single-photon emission computed tomography (SPECT) and MIBG images that heterogeneous human cardiac sympathetic innervation is accompanied by greater MIBG uptake in the anterolateral than in the inferoseptal region. This might arise partially from tissue attenuation or liver uptake of MIBG. However, animal experiments have shown a greater myocardial norepinephrine content in the LV anterolateral region, indicating increased sympathetic innervation, because norepinephrine is almost exclusively localized within adrenergic nerves. The present study found slightly greater MIBG uptake in the anterior and lateral regions than in the septal region in non-DM rats, although these differences did not reach statistical significance. Cardiac MIBG accumulation was reduced in all segments of the diabetic rat heart. However, a previous study found homogeneous cardiac MIBG accumulation in non-DM rats and a greater reduction in MIBG uptake in the inferior region of the diabetic rat heart. One study, however, found similar cardiac MIBG uptake between the anterior and inferior regions of the rat heart in a model of non-insulin dependent DM. We cannot plausibly explain these disparate results, including those of the present study. However, differences in the
LV samples investigated, such as using the basal or apical side, might be a partial explanation, because sympathetic innervation is inhomogeneous within the heart. Regional LV innervation from the base to apex forms a gradient, being highest at the base and lowest at the apex.  

Study Limitations
Some limitations should be considered when interpreting the present results. Firstly, statins might exert favorable effects on diabetic neuropathy independently of their ability to lower cholesterol. We evaluated cardiac oxidative stress, but not other mechanisms, including improved endothelial function and antiinflammatory and antithrombotic processes. Diabetic neuropathy might arise from a combination of microvascular and neural deficits, and therefore, these mechanisms combined with reduced oxidative stress might contribute to the improved cardiac MIBG accumulation in DM rats. Moreover, whether or not statins other than fluvastatin have similar effects on diabetic sympathetic neuropathy remains unknown. Secondly, we did not measure myocardial blood flow, which would influence cardiac MIBG uptake. However, a previous study has shown that myocardial blood flow is not reduced in rats with STZ-induced DM, although coronary flow reserve might be impaired in the diabetic heart.

Conclusion
Fluvastatin ameliorated cardiac sympathetic neuropathy in the diabetic rat heart in association with attenuation of increased cardiac oxidative stress. Autonomic neuropathy in diabetic patients is associated with high morbidity and mortality, so statins might help to prevent cardiovascular events and attenuate cardiac sympathetic neural dysfunction.

Acknowledgments
MIBG was generously supplied by FUJIFILM RI Pharma Co Ltd (Tokyo, Japan) and fluvastatin by Mitsubishi Tanabe Pharma Co Ltd (Osaka, Japan).

References


