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strepotozotozocin

PM2.5

8-OHdG
VEGF   NOX
Endothelin-1   IL-6
TNF-
C-reacitve protein

LDH

IL-6 8-OHdG ET-1
NOX
8-OHdG
ET-1   NOX

Recent epidemiologic studies suggest that the effect of particulate air pollution on the cardiopulmonary system is a significant public health concern. Subjects with existing respiratory and cardiovascular disease are though to be more susceptible to PM exposures. Some researchers suggest that diabetes is another sub-population at risk for PM-associated cardiovascular events. However, the biological mechanism remains unclear. The goal of this study is to use diabetic animal model to examine the relationship between PM exposure and diabetes.

We used strepotozotozocin–induced rats as diabetic animal model. Rats were exposed to $PM_{2.5}$ collected from Hsing-Chung, Taipei by a single intratracheal instillation. The pathophysiological markers were examined after PM exposure, including oxidative stress, endothelial dysfunction, acute phase and proinflammatory cytokine. The pulmonary inflammation and injury were also measured after PM exposure.

The results revealed a significant lung inflammation after PM exposure. An increased total cells and proportion of neutrophils, protein and LDH activity in bronchoalveolar lavage were observed in both non-DM and DM rats, however, diabetes did not modify the level of lung inflammation. As compared with non-DM rats, diabetic rats demonstrated significantly higher plasma 8-OHdG, IL-6 and ET-1, and lower nitrate and nitrite. The PM effect was significant in 8-OHdG, ET-1 and nitrite and nitrate in diabetic rats as compared with non-DM.

Our study found both PM and diabetes contributed to increased oxidative stress and decreased endothelial function in common pathway. Further studies are needed to clarify the mechanism.

Key words particulate matters, diabetes, cardiovascular disease.

The association between ambient particulate matter (PM) and cardiovascular diseases has been demonstrated in epidemiological studies (Pope and Dockery, 1999; Samet et al., 2000; Pope et al., 2002). Subjects with existing cardiovascular diseases including ischemic heart disease and congestive heart failure are found to be more susceptible to ambient PM exposure (Pope et al., 2002). Recently, some researchers have also suggested that diabetic patients are another sub-population at risk for PM-associated cardiovascular events (Goldberg et al., 2001; Zanobetti et al., 2001, 2002). Diabetes has been reported as an effect modifier of air pollution related hospital admissions for heart disease in elder person (Zanobetti et al., 2001), and demonstrated doubling the risk of a PM_{10}-associated cardiovascular admission compared with non-diabetics (Zanobetti et al., 2002). Diabetes mellitus is a common disease, and has been associated with cardiovascular morbidity and mortality (Resnick et al, 2001). PM is also reported to affect cardiovascular diseases (Pope et al., 2004). However, the mechanisms through which PM enhances the risks of cardiovascular disease in diabetics remain unclear. We hypothesize that diabetes and PM may share common pathway and interact in the development of cardiovascular events.

Hyperglycemia of diabetics has been associated with increased reactive oxygen species (ROS) formation (Maritim et al., 2003). Diabetic patients usually have significantly elevated concentrations of 8-OHdG in their serum (Nishikawa et al., 2003) and decreased levels of glutathione (GSH) (Dincer et al., 2002). It is proposed that increased ROS may induce inflammation in endothelium, alter endothelium function, and increase coagulability (Beckman et al., 2002). Inflammation activity also increased in individuals with diabetes, as shown by increased levels of C-reactive protein (CRP) (Jager et al., 1999; Schalkwijk et al., 1999), interleukin-6 (IL-6)(Schram et al., 2003), and tumor necrosis factor (TNF-α) (Lechleitner et al., 2000). Hyperglycemia also inhibits the production of nitric oxide (NO) by blocking eNOS synthase activation and increasing the production of ROS in endothelial and vascular smooth muscle cells (De Vriese et al., 2000). In addition to reducing concentration of NO, diabetes increases the production of vasoconstrictors, most importantly, endothelin-1 (ET-1). Reports on endothelial dysfunction in patients with diabetes have been widely studied, including increased ET-1 and decreased NO (Haak et al., 1992; Williams et al., 1996).

The exact mechanisms through which ambient PM causes cardiovascular diseases remain unclear. PM exposure is associated with

increased generation of ROS (Tao et al., 2003). It is proposed that oxidative stress in peripheral blood induced by PM may be potentially related to cardiovascular disease (Sorensen et al., 2003). PM exposure is also associated with elevated levels of C-reactive protein (Peters et al., 2001; Pope et al., 2003), enhanced production of proinflammatory cytokines (Ghio and Devlin 2001; Seaton et al., 1999; Schwartz 2001; Peters et al., 2001) and increased blood viscosity (Seaton et al., 1995; Peters et al., 1997) in epidemiological studies. In animal studies, the association between PM exposure and increased ET-1 has also been reported (Bouthillier et al., 1998; Vincent et al., 2001). It appears that PM and diabetes share common pathway in the development of cardiovascular diseases. Thus, we hypothesize exposure to PM may increase the risk of cardiovascular diseases of diabetes through the enhanced production of oxidative stress, systemic inflammation and endothelial dysfunction.

In order to determine whether PM exposure may induce synergistic effects in diabetics, we exposed streptozotocin (STZ)-induced diabetic rats to PM. Streptozotocin is a metabolite of the soil organism streptomyces achromogenes and was first reported to be diabetogenic in studies of dogs and rats in 1963 (Bell et al., 1983). Diabetes is caused by a direct toxic effect of streptozotocin on the pancreatic beta cell. After the administration of streptozotocin, there is a characteristic increase in blood glucose, which is maintained at the level of 400mg/dL or greater. This diabetic animal model has been used in many studies of diabetes pathophysiology for years (Vural et al., 2002; Ryu et al., 2003; Zang et al., 2003)

2.1 Particle collection and characterization of particles.

The ambient particles in Hsin-Chuang, Taipei were collected by a particle concentrator (Sioutas et al., 1999). The particle concentrator used virtual impactor technology in which 110 l/min flow was channeled through a saturator, cooler, impactor, and diffusion dryer to generate concentrated particles with aerodynamic diameters between 0.01 and 2.5μm. Particles were collected onto Telfon filters and the elemental compositions of the particles were determined using X-ray fluorescence (XRF, Model 6600 Jordan Valley AR, Inc., Migdal Haemek, Israel). In addition, data of water-soluble ions and carbonaceous content of $PM_{2.5}$ were obtained from Taiwan EPA supersite located at the sampling site. For these measurements, an R&P ambient carbon particulate monitor 5400 (Rupprecht & Patashnick Co., Inc., Albany, NY, USA) was used to determine the concentrations of organic and elemental carbons in $PM_{2.5}$. Meanwhile, an R&P ambient particulate nitrate monitors 8400N (Rupprecht & Patashnick Co., Inc., Albany, NY, USA) and an R&P ambient particulate sulfate monitor 8400S (Rupprecht & Patashnick Co., Inc., Albany, NY, USA) were used to determine the concentrations of $PM_{2.5}$ nitrate and sulfate. The characteristics of particles were listed in Table 1. The particulate properties represented typical urban, traffic-oriented ambient particles. All collected Teflon filters were equilibrated in 50 ± 5% relative humidity for more than 48 hours and weighed before and after air sampling to obtain particle mass. After XRF analysis, a total of six filters was submerged in endotoxin-free water and sonicated for 30 min to collect particles. These particles were pooled together before the instillation. Thus, exposed animals received same components of particles.

2.2 Diabetic animals and intratracheal instillation of PM.

Male Sprague-Dawley rats, weighing 200~250 g, were obtained from the National Laboratory Animal Breeding and Research Center, Taiwan. They were housed in plastic cages on Aspen chip bedding, and provided with Lab Diet 5001, water ad lib, except during the exposure. Animals were maintained on a 12-hour light/dark cycle at 22 ± 1°C and 55 ± 10 % relative humidity. A single intraperitoneal (IP) injection of streptozotocin (STZ, Sigma Chemical Co., 60mg/kg body weight, dissolved in citric acid buffer, pH 4.5) was administered to eight animals to induce diabetes (Bell et al., 1983), while the other eight rats were administered with citric acid as non-diabetic rats. A dose of 200μ g particles was suspended in 0.5 ml of normal saline, and sonicated for 20 min before use. The doses, which were expected to cause lung inflammation and possible subsequent systemic inflammation and endothelial

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dysfunction, were based on our experience and a previous study (Li et al., 1997). Eight rats (N=4 for diabetic and non-diabetic rats, respectively) were anesthetized with pentobarbital (50 mg/kg body weight) and then instilled with PM intratracheally. While the other eight rats (N=4 for diabetic and non-diabetic rats, respectively) were instilled with normal saline as controls. All protocols used in this experiment were approved by National Taiwan University's animal care and use committee.

2.3 Bronchoalveolar lavage analysis.

Rats were anesthetized with pentobarbital (50 mg/kg body weight) and were sacrificed 24h after intratracheal instillation. We chose 24 hours because our previous experience indicated that significant pulmonary inflammation developed at 24 hour after PM instillation, and it was hypothesized that systemic and endothelial inflammation developed following the lung inflammation. BAL fluid was collected by washing the airway with a phosphate-buffered saline solution (PBS, pH = 7.4, 28 ml/kg body weight) five times. Lavage fluid was used to determine the total number of cells and cell differential counts. Macrophage, lymphocyte, neutrophils, eosinophils and basophiles were counted under light microscopy (200 cells/slide). The remaining lavage fluid was used for the analysis of total protein and LDH activity. LDH activity was determined

by autoanalyzer at National Taiwan University Hospital. Total protein was determined using total protein assay kit (BioRad Co.)

2.4 Preparation of blood samples

A total of 15 ml whole blood was recovered from aorta. Immediately, 1 ml of whole blood was collected in citrate tube for blood glucose. 10ml whole blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes. After centrifuged at 1000 xg for 25 minutes, plasma aliquots were stored at -80 for cytokine, 8-OHdG, ET-1 and [nitrate+nitrite] analysis. The remaining 4 ml whole blood was collected in glass tube to clot for 2 hours at room temperature. After centrifuged for 20 minutes, serum samples were stored at -80 for CRP analysis.

2.5 Determination of blood glucose

Blood glucose of animals were determined using autoanalyzer (Glucometer 3, blood glucose meter; Miles Inc.) at National Taiwan University Hospital.

2.6 Determination of plasma cytokine interleukin-6 (IL-6)

Rat IL-6 in plasma was determined using ELISA kit (R&D systems, Inc.). A monoclonal antibody specific for rat IL-6 was coated on 96 well polystyrene microplate. A polyclonal antibody against rat IL-6 conjugated to horseradish peroxidase was used as rat IL-6 conjugate. Recombinant rat IL-6 ranging from 62.5 to 2000 pg/ml was used as standard. The minimum

detectable concentration of rat IL-6 in this assay ranged from 14 to 36 pg/ml. All procedures were followed the manufacturer's recommendation.

2.7 Determination of plasma cytokine tumor necrosis factor-α (TNF-α)

Rat TNF-α in plasma was determined using ELISA kit (R&D systems, Inc.). A monoclonal antibody specific for rat TNF-α was coated on 96 well polystyrene microplate. A polyclonal antibody against rat TNF-α conjugated to horseradish peroxidase was used as rat TNF-α conjugate. Recombinant rat TNF-α ranging from 12.5 to 400 pg/ml were used as standard. The minimum detectable concentration of rat TNF-α in this assay was less than 5 pg/ml. All procedures were followed the manufacturer's recommendation.

2.8 Determination of serum C-reactive protein (CRP)

Rat serum CRP was determined using the C-reactive protein kit (Helica Biosystems, Inc.). An affinity purified rabbit anti-rat CRP-IgG was precoated on a 96 well microplate. Concentrated affinity-purified horseradish peroxidase-labeled rabbit anti-rat CRP-IgG was used as conjugate. Rat serums ranging from 4.9 to 133.3 μg/ml were used as standards. The detection limit of this assay was 2.5 ng/ml. All procedures were followed the manufacturer's recommendation.

2.9 Determination of plasma 8-OHdG

Ultrafiltered plasma samples were used for the determination of 8-OHdG levels with a competitive ELISA kit (OXIS). The detection range was between 0.5 and 200 ng/ml. The 8-OHdG monoclonal antibody and plasma sample were loaded at 50 μl on a microtiter plate which had been coated with 8-OHdG. All procedures were followed the manufacturer's recommendation.

2.10 Determination of plasma endothelin-1 (ET-1)

Rat ET-1 was determined using an ELISA kit (R&D systems, Inc.). A murine monoclonal antibody against ET-1 was precoated on microplate, and monoclonal antibody ET-1 conjugated to HRP was used as ET-1 conjugate. Synthetic human ET-1 ranging from 0.32 to 1000 pg/ml were used as standards. The minimum detectable dose of ET-1 was less than 0.16 pg/ml. All procedures were followed the manufacturer's recommendation.

2.11 Determination of plasma [nitrate+nitrite]

The NO production was determined in plasma using Nitric Oxide Synthase Assay Kit, Colorimetric (CALBIOCHEM Inc). The final products of NO in vivo are nitrite $[NO_2^-]$ and nitrate $[NO_3^-]$. This assay uses the sum of $[NO_2^-]$ and $[NO_3^-]$ as the index of total NO production. A total of 40 μl of ultrafiltered plasma sample reacted with 1mM NADPH and nitrate reductase for 60 minutes at room temperature. Then 10μl of cofactors and LDH was added and incubated for 20 minutes. Subsequently, Griess reagent R1 and R2

were added to develop color for 10 minutes, and the absorbance was read at 540 nm. Determination of [nitrate+nitrite] concentration was adjusted with the standard curve of nitrate according to the manufacturer's equation.

2.12 Statistical analysis.

The experiment design of this study is a 2^2 factorial design with 4 replicates for each treatment combination. The 2 factors are PM exposure and diabetes. The 2 levels for each factor are yes and no. Multiple response variables are measured simultaneously for each run. The 4 observations of each response variable in each treatment combination were first summarized by its mean and standard deviation to get an insight of the responses. We then used Wilcoxon rank-sum test to examine response difference between any two of the 4 treatment combinations because of only 4 observations in each treatment combination. Traditionally, the observations of a response variable in the factorial design are often fitted to a general linear model to estimate its main effects of the factors and interactions. Specifically, the 16 observations denoted by y_{ijk}, $i, j = 1, 2$ and $k = 1, 2, 3, 4$ of a response variable were further described by the linear statistical model

$$y_{ijk} = \mu + \alpha P_i + \beta D_j + \gamma PD_{ij} + \varepsilon_{ijk} \quad ,$$

where $P_i = 1$ when level i is the group exposed to PM and 0, otherwise;

$D_j = 1$ when level j is the diabetes group and 0, otherwise; $PD_{ij} = 1$ when $P_i = D_j$ and 0, otherwise. The error term is assumed to be independently and normally distributed. The parameter μ is for overall mean of the response variable. The coefficients, α, β and γ in this model represent PM exposure effect, diabetes effect and interaction between PM exposure and diabetes. We fit this general linear model and calculate the maximum likelihood estimates of these coefficients and their standard errors. Type I error was set at 0,05 for significance. The t statistic was used for testing significance of the coefficients. The residuals from individual fits were also examined. If diagnostic plots for the residuals show departures from the general linear models, we would find a proper transformation on the response variable or use Wilcoxon rank-sum tests instead for testing the 2 main effects and no testing for the interaction.

3.1 Characteristics of study animals and effects of diabetes.

Characteristics of study animals were described in Table 2. Body weight of diabetic rats was lower than that of non-diabetic rats (397.5g vs. 483.1g). The mean plasma glucose level was 163 mg/dl in non-diabetic rats, and 448.2

mg/dl in diabetic rats ($p < 0.05$).

Effects of diabetes on different parameters in bronchoalveolar lavage were described in Table 2. Diabetes had no effect on total cells, percentage of neutrophils, total protein and LDH activity in BAL (Table 3). In plasma analysis, diabetic rats demonstrated significantly greater 8-OHdG generation (6.2 vs. 6.8 ng/ml, $p < 0.05$) and cytokine IL-6 (42.3 vs. 66.0 pg/ml, $p < 0.05$) as compared with non-diabetic rats, (Table 3). Furthermore, diabetic rats had significantly increased level of plasma ET-1 (2.1 vs. 2.8 pg/ml, $p < 0.05$), and decreased level of plasma [nitrate+nitrite] (107.8 vs. 87.0 μM, $p < 0.05$). There was no observable change in TNF-α and CRP between diabetic and non-diabetic rats.

3.2 Effects of PM exposure.

In non-diabetic rats, PM caused significant increases in total cells and proportion of neutrophils in bronchoalveolar lavage (Table 3, $p < 0.05$). Elevated total protein and LDH activity in bronchoalveolar lavage were also observed after PM exposure ($p<0.05$). Plasma 8-OHdG level also showed an increase after PM exposure (Table 4, $p = 0.08$). Similar finding were observed in plasma cytokine IL-6, TNF-α, and serum CRP ($p<0.05$). Furthermore, PM exposure caused a significant reduction of plasma [nitrate+nitrite] ($p<0.05$). However, there was no significant PM effect on plasma ET-1 in non-diabetic rats.

In STZ-diabetic rats, significant increases in pulmonary inflammation and injury markers were observed after PM exposure (Table 3, $p<0.05$). Plasma 8-OHdG and cytokine TNF-α significantly increased in diabetic rats after exposure to PM (Table 4, $p < 0.05$). There were no significant alteration in cytokine IL-6 and CRP after PM exposure. In assessing endothelial function, we found a significant elevation of plasma ET-1 ($p < 0.05$) and a decrease in plasma [nitrate+nitrite] ($p = 0.08$) after exposure to PM.

3.3 Interaction between PM exposure and diabetes.

When the PM effects were further compared between diabetic and non-diabetic rats, we found that increases of 8-OHdG and ET-1 were more prominent in diabetic rats. For 8-OHdG generation, diabetic rats exposed to PM demonstrated a 15.6 % increase; however, non-diabetic rats exposed to PM showed only 4.0 % increase. A 40.3 % increase in plasma ET-1 after PM exposure was observed in diabetic rats, while there was only a 2.6 % increase in plasma ET-1 after PM exposure in non-diabetic rats. General linear model was further used to test the interaction between diabetes and PM. We found there were interactions on 8-OHdG (Table 5, $p<0.01$) and ET-1 ($p=0.08$).

In this study, we compared the influence of PM on parameters involved in cardiovascular disease between diabetic and healthy rats. Our results showed that diabetic rats were associated with increased 8-OHdG, IL-6 and ET-1, but decreased [nitrate+nitrite]. In non-diabetic rats, PM exposure was also associated with increased plasma 8-OHdG, IL-6, TNF-α and CRP, but decreased [nitrate+nitrite]. Interestingly, we found that increases of 8-OHdG and ET-1 after PM exposure were more prominent in diabetic rats as compared to non-diabetic rats. Statistical analysis further indicated that there were interactions between diabetes and PM on plasma 8-OHdG and ET-1.

Diabetes mellitus is a highly prevalent chronic illness, and affects approximately 100 million people worldwide (Amos et al., 1997). It has been proposed that endothelial cell inflammation, excessive ROS and endothelial dysfunction may play important roles in diabetes-related cardiovascular diseases (Bechman et al., 2002). Our study has demonstrated that STZ-diabetic rats had significantly increased plasma ROS, cytokine IL-6 and ET-1, and decreased NO. We also found a borderline increase in serum CRP in STZ-diabetic rats. The results were consistent with previously studies (Ryu et al., 2003; Zhang et al., 1999; Pickup 2004; Vural et al., 2002). Although diabetic rats were found to have endothelial dysfunction, we did not

observe any lung inflammation or injury in diabetic rats without PM treatment. It appears that STZ-diabetic rat is a sensitive model for studying cardiovascular diseases in diabetics.

In this study, we found significant increase of total cells, percentage of neutrophil, LDH and protein in BAL after PM exposure in non-diabetic rats. These findings are consistent with previous studies (Gordon et al., 1998; Clarke et al., 1999; Lei et al., 2003). We also observed elevated PM-induced 8-OHdG and systemic inflammation, including IL-6, TNF-α and CRP. The oxidative capacity of PM may be attributed to its transition metal constituents (Prahalad et al., 1999; Clarke et al., 2000). The concentration of transition metals on PM collected in our studies was much lower than that in other CAPs studies conducted in the United States, therefore we speculate that the role of transition metals may be less significant on PM-related effects in this study. Interestingly, previous study has shown that ultra-fine carbon black induces significant higher ROS compared to fine carbon black under the same mass concentration (Wilson et al., 2002). Because our particles collected from particle concentrator with size ranging between 0.01 and 2.5μm, it is very likely that ultrafine particles account for, at least partly, the oxidative stress and inflammation. The elevations of oxidative stress may subsequently cause increases in IL-6 leading to lung

inflammation (Tao et al., 2003). IL-6 has been reported to induce CRP in liver (Blake and Ridker, 2002). Recent studies have also demonstrated the CRP is an independent risk factor for cardiovascular diseases (Backes et al., 2004), and elevated CRP has been reported to be associated with PM exposure in the elderly subjects (Pope et al., 2004). As for TNF-α, it is most likely secreted by alveolar macrophage after PM exposure (Beacher et al., 2001). The effects of PM on lung inflammation and pro-inflammatory cytokines were consistent with previous studies.

NO exerts multiple modulating effects on inflammation and plays a key role in the regulation of vascular tone (Guzik et al, 2003). In our study, a significant decrease of nitrate and nitrite levels, an indicator of NO production, after PM exposure was observed in our study in healthy rats. A recent study found a decrease in plasma NO level after instillation exposure to PM from Ottawa, although no statistical significance was reached (Ulrich et al., 2002). NO may be formed by inducible NO synthase (iNOS) in macrophages and other cells (Guzik et al, 2003). A previous study reported an increased production of iNOS-dependent NO by alveolar macrophage following dust exposure (Blackford et al., 1997). Although NO decrease may be resulted from the PM effects in airway, it is not clear if decreased NO is also resulted from the PM effects on endothelium.

NO may be formed from endothelial NOS (eNOS). It has been proposed that ROS may inhibit the activity of eNOS, which lead to reduction of NO synthesis (Beckman et al., 2002). It is plausible that NO decrease is caused by ROS induced by penetrating PM (Nemmar et al., 2001, 2002), or indirectly by ROS formed in the lungs.

In our study, there was no increase for plasma ET-1 after PM instillation in non-diabetic rats. Data on the relationship between PM exposure and circulating ET-1 were limited. Recent studies reported an increase in plasma ET-1 after PM instillation in healthy rats, but they did not reach a significant level (Bouthillier et al., 1998; Ulrich et al., 2002). Another study proposed that concentrated ambient particles-induced vasoconstriction of small pulmonary arteries in rats might be due to elevated ET-1 production in pulmonary vessel endothelium (Batalha et al., 2002). Similar to NO reduction, PM may cause ET-1 increase through direct effect on endothelium after penetrating into the circulation, or indirectly through lung inflammation. It seems PM with certain components and size may cause alterations in NO and ET-1.

In our study, we observed PM and diabetic status individually altered oxidative stress and endothelial dysfunction, and the greatest effects were observed in diabetic rats with PM exposure. Significant synergistic interactions between PM exposure and

diabetes on 8-OHdG and ET-1 levels were observed. In diabetes, hyperglycemia engenders adverse metabolic events with the generation of ROS in endothelial cells. We speculate that excessive ROS generation may affect the antioxidant capacity in endothelium, and lead to further synergistic interaction on ROS generation after PM exposure. Subsequently, excessive ROS may impair endothelial function through increased ET-1. Evidence suggests a pathophysiologic role of ET-1 in diseases affecting the cardiovascular system, including hypertension, cerebrovascular disease and heart failure (Schiffrin et al., 1997). In patients with heart failure, plasma ET-1 levels are two- to fourfold elevated and correlate with the severity of the disorder (Packer et al., 1993). Clinical observations also support a pathophysiologic role of ET-1 in coronary atherosclerosis. Increased plasma ET-1 concentrations were observed after myocardial infarction and persistent elevations of ET-1 predict an increased mortality within the subsequent 12 months (Lerman et al., 1991). In our earlier discussion, we have demonstrated that PM can increase the oxidative stress and cause endothelial dysfunction. Thus, it is plausible that PM exposure may enhance the risk of cardiovascular diseases in diabetes through the possible interaction between PM and diabetes in endothelium.

Plasma 8-OHdG was used as an indicator of oxidative stress in this study. Since 8-OHdG is an excision repair product of oxidative DNA damage, such damage in other tissues in addition to lung and endothelium may also contribute to the increase of plasma 8-OHdG. Previous study has showed that treatment of STZ resulted in elevated DNA damage in liver and kidney (Imaeda et al., 2002) and indirectly contribute to increased plasma 8-OHdG level. However, this did not affect the results in this study, because PM effect on plasma 8-OHdG levels was compared in diabetic rats and non-diabetic rats, respectively. In addition, plasma 8-OHdG levels may also be affected by a decrease in clearance because of kidney disease in diabetics. Previous studies have reported an increased glomerular filtration rate, decreased renal plasma flow, increased filtration fraction and increased blood urea nitrogen in STZ-diabetic rats (Carney et al., 1979; Bell and Hye, 1983; Somova et al., 1988). Again, this did not affect the results in this study, because PM effect on plasma 8-OHdG levels was compared in diabetic rats and non-diabetic rats, respectively.

Inflammation is also a crucial factor in cardiovascular disease and PM-induced effect. In this study, we did not observe significantly enhanced IL-6, TNF-α and CRP in diabetic rats exposed to PM. Higher production of IL-6, TNF-α and CRP have been reported in diabetic patients and after PM exposure

(Spranger et al., 2003). However, it is not clear why there is no interaction between diabetes and PM on inflammatory markers. One explanation is that cytokines have many different sources other than lung and endothelium (Rankin 2004). The other explanation is that these cytokines are independently produced in lung and endothelium. However, the exact mechanisms need further study.

The limitation of this study is that instilled doses of particles were not adjusted by the body weight, although diabetic rats had lower body weight as compared to non-diabetic rats. However, the PM effects in diabetic and non-diabetic groups are less likely to be affected, because the doses were proportionally higher in diabetic group. The other limitation of this study was the small number of animals, although the statistical analysis has been properly conducted. Thus, further studies are needed to confirm our findings.

Our results revealed that PM and diabetic status individually altered oxidative stress and endothelial dysfunction, and the greatest effects were observed in diabetic rats with PM exposure. We conclude that PM exposure may enhance the risk of cardiovascular diseases through the possible interaction between PM and diabetes in endothelium. These findings provide further supports for previous epidemiological studies. In this study, we also demonstrate that STZ-diabetic rat is a useful model in studying PM-related cardiovascular effects.

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Amos, A.F., McCatry, D.J., Zimmet, P. 1997. The rising global burden of diabetes and its complications: estimates and projections to the year 2010. Diabet. Med. 14 (suppl 5), S1-S85.

Backes, J.M., Howard, P.A., Moriarty, P.M. 2004. Role of C-reactive protein in cardiovascular disease. Ann. Pharmacother. 38, 110-118.

Batalha, J.R., Saldiva, P.H., Clarke, R.W., Coull, B.A., Stearns, R.C., Lawrence, J., Murthy, C.G., Koutrakis, P., Godleski, J.J. 2002. Concentrated ambient air particles induce vasoconstriction of small pulmonary arteries in rats. Environ. Health. Perspect. 110,1191-1197

Becher, R., Hetland, R.B., Refsnes, M., Dahl, J.E., Dahlman, H.J., Schwarze, P.E. 2001. Rat lung inflammatory responses after in vivo and in vitro exposure to various stone particles. Inhal. Toxicol. 13, 789-805.

Beckman, J.A., Creager. M.A., Libby, P. 2002. Diabetes and atherosclerosis: epidemiology, pathophysiology,

and management. JAMA. 2872570-2581.

Bell, R.H., Hye, R.J. 1983. Animal models of diabetes mellitus: physiology and pathology. J. Surgical. Res. 35, 433-460.

Blackford, J.A., Jones, W., Dey, R.D., Castranova. V. 1997. Comparison of inducible nitric oxide synthase gene expression and lung inflammation following intratracheal instillation of silica, coal, carbonyl iron, or titanium dioxide in rats. J. Toxicol. Environ. Health. 51, 203-218.

Blake, G.J., Ridker, P.M. 2002. Inflammatory bio-markers and cardiovascular risk prediction. J. Intern. Med. 252, 283-294.

Bouthillier, L., Vincent, R., Goegan, P., Adamson, I.Y., Bjarnason, S., Stewart, M., Guenette, J., Potvin, M., Kumarathasan, P. 1998. Acute effects of inhaled urban particles and ozone: lung morphology, macrophage activity, and plasma endothelin-1. Am. J. Pathol. 153, 1873-1884.

Carney, S.L., Wong, N.L., Dirks, J.H. 1979. Acute effects of streptozotocin diabetes on rat renal function. J. Lab. Clin. Med. 93, 950-961.

Clarke, R.W., Catalane, P.J., Koutrakis, P., Krishna, M., Sioiutas, C., Paulauskis, S.J. 1999. Urban air particulate inhalation alters pulmonary function and induces pulmonary inflammation in a rat model of chronic bronchitis. Inhal. Toxicol. 11, 637-656.

Clarke, R.W., Coull, B., Reinisch, U., Catalano, P., Killingsworth, C.R., Koutrakis, P., Kavouras, I., Murthy, G.G., Lawrence, J., Lovett, E., Wolfson, J.M., Verrier, R.L., Godleski, J.J. 2000. Inhaled concentrated ambient particles are associated with hematologic and bronchoalveolar lavage changes in canines. Environ. Health. Perspect. 108, 1179-87.

De Vriese, A.S., Verbeuren, T.J., Van de Voorde, J., Lameire, N.H., Vanhoutte, P.M. 2000. Endothelial dysfunction in diabetes. Br. J. Pharmacol. 130, 963-974.

Dincer, Y., Akcay, T., Alademir, Z., Ilkova, H. 2003. Assessment of DNA base oxidation and glutathione level in patients with type 2 diabetes. Mutat. Res. 525, 129-130.

Ghio, A.J., Devlin, R.B. 2001. Inflammatory lung injury after bronchial instillation of air pollution particles. Am. J. Respir. Crit. Care. Med.164,704-708.

Goldberg, M.S., Burnett, R.T., Bailar, J.C. 2001. The association between daily mortality and ambient air

particle pollution in Montreal, Quebec. 2. Cause-specific mortality. Environ. Res. 86, 26-36.

Gordon, T., Nadziejko, C., Schlesinger, R., Chen, L.C., 1998. Pulmonary and cardiovascular effects of acute exposure to concentrated ambient particulate matter in rats. Toxicol. Letters. 96, 97, 285-288.

Guzik, T.J., Korbut, R., Adamek-Guzik, T. 2003. Nitric oxide and superoxide in inflammation and immune regulation. J. Physiol Pharmacol. 54, 469-487.

Imaeda, A., Kaneko, T., Aoki, T., Kondo, T., Nagase, H. 2002. DNA damage and the effect of antioxidants in strepotozotocin-treated mice. Food. Chem. Toxicol. 40, 979-987.

Haak, T., Jungmann, E., Felber, A., Hillmann, U., Usadel, K.H. 1992. Increased plasma levels of endothelin in diabetic patients with hypertension. Am. J. Hypertens. 5,161-166.

Jager, A., van Hinsbergh, V.W., Kostense, P.J., Emeis, J.J., Yudkin, J.S., Nijpels, G., Dekker, J.M., Heine, R.J., Bouter, L.M., Stehouwer, C.D. 1999. von Willebrand factor, C-reactive protein, and 5-year mortality in diabetic and nondiabetic subjects: the Hoorn Study. Arterioscler. Thromb. Vasc. Biol. 19, 3071-3078.

Lechleitner, M., Koch, T., Herold, M., Dzien, A., Hoppichler, F.. 2000. Tumour necrosis factor-alpha plasma level in patients with type 1 diabetes mellitus and its association with glycaemic control and cardiovascular risk factors. J. Intern. Med. 248, 67-76.

Lerman, A., Edwards, B.S., Hallett, J.W., Heublein, D.M., Sanberg, S.M., Burnett, J.C. 1991. Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. N. Engl. J. Med. 325, 997-1001.

Li, X.Y., Gilmour P.S., Donaldson, K., MacNee, W. 1997. Environ. Health Perspect. 105, 1279-1283.

Maritim, A.C., Sanders, R.A., Watkins, J.B. 2003. Diabetes, oxidative stress, and antioxidants: a review. J. Biochem. Mol. Toxicol. 17, 24-38.

Michael, J.R., Markewitz, B.A. 1996. Endothelins and the lung. Am. J. Respir. Crit. Care. Med. 154, 555-581.

Nemmar, A., Vanbilloen, H., Hoylaerts, M.F., Hoet, P.H., Verbruggen, A., Nemery, B. 2001. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. Am. J. Respir. Crit. Care. Med. 164, 1665-1668.

Nemmar, A., Hoet, P.H., Vanquickenborne, B., Dinsdale, D.,

Thomeer, M., Hoylaerts, M.F., Vanbilloen, H., Mortelmans, L., Nemery, B. 2002. Passage of inhaled particles into the blood circulation in humans. Circulation. 105, 411-414.

Nishikawa, T., Sasahara, T., Kiritoshi, S., Sonoda, K., Senokuchi, T., Matsuo, T., Kukidome, D., Wake, N., Matsumura, T., Miyamura, N., Sakakida, M., Kishikawa, H., Araki, E. 2003. Evaluation of urinary 8-hydroxydeoxy-guanosine as a novel biomarker of macrovascular complications in type 2 diabetes. Diabetes. Care. 26, 1507-1512.

Packer, R., Bergler-Klein, J., Globits, S., Teufelsbauer, H., Schuller, M., Krauter, A., Ogris, E., Rodler, S., Wutte, M., Hartter, E. 1993. Plasma big endothelin-1 concentrations in or congestive heart failure patients with or without systemic hypertension. Am. J. Cardiol. 71, 1293-1299.

Park, K.S., Kim, J.H., Kim, M.S., Kim, J.M., Kim, S.K., Choi, J.Y., Chung, M.H., Han, B., Kim, S.Y., Lee, H.K. 2001. Effects of insulin and antioxidant on plasma 8-hydrozyguanine and tissue 8-hydrozyguanosine in streptozotocin-induced diabetic rats. Diabetes, 50, 2837-2841.

Peters, A., Doring, A., Wichmann, H.E., Koenig, W. 1997. Increased plasma viscosity during the1985 air pollution episode: a link to mortality? Lancet 349, 1582-1587.

Peters, A., Frohlich, M., Doring, A. 2001. Particulate air pollution is associated with an acute phase response in men: results from the MONICA-Augsbrug study. Eur. Heart. J. 22, 1198-1204.

Pickup, J.C. 2004. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes. Care. 27, 813-823.

Pope, III C.A., Dockery, D.W. 1999. Epidemiology of particle effects. In: Air Pollution and Health (Holgate ST, Samet JM, Koren HS, Maynard RL, eds.). London: Academic Press 673-705.

Pope, III C.A., Burnett, R.T., Thun, M.J., Calle, E.E., Krewski, D., Ito, K. 2002.Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution, JAMA. 287, 1132-1141.

Pope, III C.A., Hansen, M.L., Long, R.W., Nielsen, K.R., Eatough, N.L., Wilson, W.E., Eatough, D.J. 2004. Ambient particulate air pollution, heart rate variability, and blood markers of inflammation in a panel of elderly subjects. Environ. Health. Perspect. 112, 339-345.

Prahalad, A.K., Soukup, J.M., Inmon, J.,

Willis, R., Ghio, A.J., Becker, S., Gallagher, J.E. 1999. Ambient air particles: effects on cellular oxidant radical generation in relation to particulate elemental chemistry. Toxicol. Appl. Pharmacol. 158, 81-91.

Rankin, J.A. 2004. Biological mediators of acute inflammation. AACN Clin Issues. 15, 3-17.

Resnick, H.E., Howard, B.V. 2002. Diabetes and cardiovascular disease. Annu. Rev. Med. 53, 245-267.

Ryu, J.K., Kim, D.J., Lee, T., Kang, Y.S., Yoon, S.M., Suh, J.K. 2003. The role of free radical in the pathogenesis of impotence in streptozotocin-induced diabetic rats. Yonsei, Med. J. 44, 236-241,

Samet, J.M., Dominici, F., Curriero, F.C., Coursac, I., Zeger, S.L. 2000. Fine particulate air pollution and mortality in 20 U.S. cities, 1987-1994, N. Engl. J. Med. 343, 1742-1749.

Schalkwijk, C.G., Poland, D.C., van Dijk, W., Kok, A., Emeis, J.J., Drager, A.M., Doni, A., van Hinsbergh, V.W., Stehouwer, C.D. 1999. Plasma concentration of C-reactive protein is increased in type I diabetic patients without clinical macroangiopathy and correlates with markers of endothelial dysfunction: evidence for chronic inflammation.

Diabetologia. 42, 351-357

Schiffrin, E.L., Intengan, H.D., Thibault, G., .Touyz, R.M. 1997. Clinical significance of endothelin in cardiovascular disease. Curr. Opin. Cardiol. 12, 354-367.

Schram, M.T., Chaturvedi, N., Schalkwijk, C., Giorgino, F., Ebeling, P., Fuller, J.H., Stehouwer, C.D., EURODIAB Prospective Complications Study. 2003. Vascular risk factors and markers of endothelial function as determinants of inflammatory markers in type 1 diabetes: the EURODIAB Prospective Complications Study. Diabetes. Care.26, 2165-2173.

Schwartz, J. 2001. Air pollution and blood markers of cardiovascular risk. Environ. Health. Perspect. 109, 405-409.

Seaton, A., MacNee, W., Donaldon, K., Godden, D. 1995. Particulate air pollution and acute health effects. Lancet 345, 176-178.

Seaton, A., Soutar, A., Crawford, V., Elton, R., McNerlan, S., Cherrie, J., Watt, M., Agius, R. 1999. Stout R.Particulate air pollution and the blood. Thorax. 54, 1027-1032.

Sioutas, C., Kim, S., Chang, M. 1999. Development and evaluation of a prototype ultra-fine particle concentrator, J. Aerosol. Med 30,

1001-1017.

Somova, L., Dashev, G., Doncheva, M., Vassileva, M. 1988. Pathogenesis of cardiovascular disorders in streptozotocin-induced diabetes in rat. I. Cardiovascular, renal and morphologic changes in different stages of diabetes. Acta. Physiol. Pharmacol. Bulg. 14, 46-56.

Sorensen, M., Daneshvar, B., Hansen, M., Dragsted, L.O., Hertel, O., Knudsen, L., Loft, S. 2003. Personal PM2.5 exposure and markers of oxidative stress in blood. Environ. Health. Perspect. 111, 161-166.

Spranger, J., Kroke, A., Mohlig, M., Hoffmann, K., Bergmann, M.M., Ristow, M., Boeing, H., Pfeiffer, A.F. 2003. Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Diabetes. 52, 812-817.

Tao, F., Gonzalez-Flecha, B., Kobzik, L. 2003. Reactive oxygen species in pulmonary inflammation by ambient particulates. Free Radical biology and medicine 35, 327-340.

Ulrich, M.M., Alink, G.M., Kumarathasan, P., Vincent, R., Boere, A.J., Cassee, F.R. 2002. Health effects and time course of particulate matter on the cardiopulmonary system in rats with lung inflammation. J. Toxicol. Environ. Health. A 65, 1571-1595.

Vincent, R., Kumarathasan, P., Goegan, P., Bjarnason, S.G., Guenette, J., Berube, D., Adamson, I.Y., Desjardins, S., Burnett, R.T., Miller, F.J., Battistini, B. 2001. Inhalation toxicology of urban ambient particulate matter: acute cardiovascular effects in rats. Res. Rep. Health. Eff. Inst. (104):5-54; discussion 55-62.

Vural, P., Cevik, A., Curgunlu, A., Canbaz, M. 2002. Effects of diabetes mellitus and acute hypertension on plasma nitric oxide and endothelin concentrations in rats. Clin. Chim. Acta. 320, 43-47.

Williams, S.B., Cusco, J.A., Roddy, M.A., Johnstone, M.T., Creager, M.A. 1996. Impaired nitric oxide-mediated vasodilation in patients with non-insulin-dependent diabetes mellitus. J. Am. Coll. Cardiol. 27, 567-574.

Wilson, M.R., Lightbody, J.H., Donaldson, K., Sales, J., Stone, V. 2002. Interactions between ultrafine particles and transition metals in vivo and in vitro. Toxicol. Appl. Pharmacol. 184, 172-179

Zang, L., Zalewski, A., Liu, Y., Mazurek, T., Cowan, S., Martin, J.L., Hofmann, S.M., Vlassara, H., Shi,

Y. 2003. Diabetes-induced oxidative stress and low-grade inflammation in porcine coronary arteries. Circulation. 108, 472-478.

Zanobetti, A., Schwartz, J., Gold, D.R. 2001. Are diabetes more susceptible to the health effects of airborne particles? Am. J. Respir. Crit. Care. Med. 164, 831-833.

Zanobetti, A., Schwartz, J. 2002. Cardiovascular damage by airborne particles: are diabetes more susceptible? Epidemiology 13, 588-592.

Table legends

Table 1 Characterization of fine particles instilled

Table 2 Characteristics of study animals. * Mean $\pm$ standard deviation ; [a] $p<0.05$ as compared to non-diabetic rats with saline exposure; [b] $p<0.05$ as compared to STZ-diabetic with saline exposure.

Table 3 Bronchoalveolar lavage analyses in rats. * Mean $\pm$ standard deviation; [a] $p<0.05$ as compared to non-diabetic rats with saline exposure; [b] $p<0.05$ as compared to STZ-diabetic with saline exposure.

Table 4 Plasma and serum analysis of markers of oxidative stress, inflammation and endothelial function in rats * Mean $\pm$ standard deviation. [a] $p<0.05$ as compared to non-diabetic rats with saline exposure; [b] $p<0.05$ as compared to STZ-diabetic with saline exposure; [c] $p = 0.08$ as compared to non-diabetic rats with saline exposure.

Table 5 Interaction between PM exposure and diabetes in plasma 8-OHdG and ET-1.

Table 1 Characterization of fine particles instilled

Component	Concentration
Elemental components from XRF analysis	
Potassium	8.0%
Sulfur	1.5%
Aluminum	1.5%
Iron	0.9%
Phosphate	0.9%
Calcium	0.5%
Silicon	0.7%
Zinc	0.3%
Tungsten	0.04%
Vanadium	0.01%
Manganese	0.01%
Components of $PM_{2.5}$ ($\mu g/m^3$) from EPA supersite	
Organic carbon	9.8 (2.4)
Elemental carbon	3.6 (3.2)
Sulfate	4.8 (1.2)
Nitrate	6.3 (3.4)

* mean (SD)

Table 2 Characteristics of study animals

	Non-diabetic rats		STZ-diabetic rats	
	Saline	PM	Saline	PM
N	4	4	4	4
Body weight (g)	487.4±29.5 *	476.7±46.1	385±37.8[a]	410±25.8
Blood glucose (mg/dL)	166.7±10.9	155.3±10.7	467.7±78.9[a]	435.5±63.5[b]

* Mean $\pm$ standard deviation

[a] $p < 0.05$ as compared to non-diabetic rats with saline exposure.

[b] $p < 0.05$ as compared to STZ-diabetic with saline exposure.

Table 3 Bronchoalveolar lavage analyses in rats

	Non-diabetic rats		STZ-diabetic rats	
	Saline	PM	Saline	PM
N	4	4	4	4
Total number of cells (x 10^4 cell)	60.4±11.8*	120.5±12.5 [a]	54.2±10.2	136.5±19.3 [b]
Percentage of neutrophils	7.5±3.2	43.7±12.1 [a]	9.2±4.7	36.5±2.4 [b]
Total protein (mg/L)	400.7±21.3	612.1±37.5 [a]	362.4±50.8	620.5±57.2 [b]
LDH activity (U/L)	17.1±13.2	81.2±19.3 [a]	22.5±14.7	78.3±20.4 [b]

* Mean ± standard deviation

[a] p<0.05 as compared to non-diabetic rats with saline exposure.

[b] p<0.05 as compared to STZ-diabetic with saline exposure.

Table 4 Plasma and serum analysis of markers of oxidative stress, inflammation and endothelial function in rats

	Non-diabetic rats		STZ-diabetic rats	
	Saline	PM	Saline	PM
N	4	4	4	4
Oxidative stress				
Plasma 8-OHdG (ng/ml)	6.2±0.1*	6.4±0.1 [c]	6.8±0.1 [a]	7.6±0.3 [a b]
Inflammation				
Serum CRP (µg/ml)	289.7±55.6	379.7±46.9 [a]	355.5±48.9	381.1±30.2 [a]
Plasma IL-6 (pg/ml)	42.3±6.2	71.6±0.3 [a]	66.0±11.7 [a]	90.3±29.5 [a]
Plasma TNF-α (pg/ml)	7.7±0.8	9.7±0.8 [a]	7.8±0.9	10.3±1.1 [a b]
Endothelial function				
Plasma ET-1 (pg/ml)	2.1±0.1	2.2±0.2	2.8±0.3 [a]	3.2±0.1 [a b]
Plasma NO (µM)	107.8±10.4	89.5±2.8 [a]	87.0±11.0 [a]	71.5±3.8 [a]

* Mean ± standard deviation

[a] $p < 0.05$ as compared to non-diabetic rats with saline exposure.

[b] $p < 0.05$ as compared to STZ-diabetic with saline exposure.

[c] $p = 0.08$ as compared to non-diabetic rats with saline exposure.

Table 5 Interaction between PM exposure and diabetes in plasma 8-OHdG and ET-1

	Plasma 8-OHdG			Plasma ET-1		
	Estimate value	Standard error	p-value	Estimate value	Standard error	p-value
Intercept	5.89	0.085	<0.01	1.92	0.092	<0.01
PM effect	0.49	0.085	<0.01	0.24	0.092	<0.05
Diabetes effect	0.91	0.085	<0.01	0.92	0.092	<0.01
Interaction between PM and diabetes	0.31	0.085	<0.01	0.17	0.092	0.08