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Originals

HMG-CoA Reductase Inhibitors Suppress High Glucose-induced Excessive O_2^- production in J-774 Cells

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SUMMARY

Statins (HMG-CoA reductase inhibitors) have so-called pleiotropic effects, which directly reduce neoinitial inflammation of atherosclerosis, and one possible mechanism is the attenuation of oxidative stress. Although an increase in oxidant stress is suggested to cause and aggravate arteriosclerosis in diabetes, the origin of oxidant stress and effects of statins on the oxidant stress in diabetes are not clearly delineated. We evaluated in this study the effect of high glucose on superoxide anion (O_2^-) production in the J-774 macrophage-like cell line, and the effect of various statins (cerivastatin, fluvastatin, and nisvastatin) on it. The basal and 12-O-tetradecanoylphorbol 13-acetate (TPA)-stimulated O_2^- productions were measured by chemiluminescence (CL) amplified with a Cypridina luciferin analog. Both basal CL and TPA-stimulated CL (TPA-CL) in J-774 cells cultured with high glucose were apparently increased in dose and time dependent manners, and the increments were clearly suppressed by a NADPH oxidase inhibitor (diphenyleneiodonium chloride) or a protein kinase C inhibitor (GF-109803X). Three statins significantly inhibited the high glucose-induced excessive O_2^- production in a dose dependent manner. Furthermore, co-incubation with mevalonic acid and the metabolites, geranylgeranyl pyrophosphate and farnesyl pyrophosphate, partially prevented the statin-induced suppression of TPA-CL. These data suggest that in J-774 cells high glucose causes excessive O_2^- production through NADPH oxidase and protein kinase C pathways, and statins suppress the excessive O_2^- generation. This effect of statins could be, in part, dependent on the inhibition of synthesis of isoprenoid intermediates. Statins may be useful as a drug to prevent arteriosclerosis by inhibiting oxidative stress in poorly controlled diabetic patients.

Key Words : HMG-CoA inhibitors, superoxide anion, macrophage, high glucose

INTRODUCTION

The risk of atherosclerotic vascular disease is markedly increased in patients with diabetes[1]. Many risk factors are common in patients with diabetes, including insulin resistance, hypertension, and dyslipidemia and these risk factors are supposed to participate in the overall process of accelerated atherogenesis. Considerable attention has been focused on the role of "oxidative stress (an excessive production of reactive oxygen species)" in atherogenesis[2]. An enhanced oxidative stress has been observed in diabetes as indicated by increased free radical production[3], lipid peroxidation, and diminished antioxidant status[4]. There are a number of tenable biochemical pathways connecting hyperglycemia with enhanced production of reactive oxygen species (e.g. advanced glycation end product formation, altered polyol pathwa activity, activation of NADPH oxidase). Although the origin of reactive oxygen species is not yet completely clear, monocytes from diabetic patients have enhanced production of toxic oxygen products[3,6] and the oxidative stress in leukocytes was recently suggested to be one of the risk factors for cardiovascular disease in diabetes[7].

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The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) can achieve relatively large reduction in plasma cholesterol levels and represent an established class of drugs for the treatment of hypercholesterolemia[8]. Several clinical trials have demonstrated that statins can ameliorate vascular atherosclerosis, and reduce morbidity and mortality in patients with coronary artery disease symptoms. Interestingly, there is now a variety of clinical and experimental evidence to show that some statins can interfere with major events involved in the formation of atherosclerotic lesions, independently of their hypolipidemic properties[9, 10]. Lipid-lowering clinical trials suggest that despite comparable reduction in serum cholesterol levels, the risk of cardiovascular events in statin-treated patients is lower than with other agents or methods used to decrease serum cholesterol levels[11]. This profound biological effect of statins is a so-called pleiotropic effect[12], and the attenuation of oxidative stress is believed to be one of the pleiotropic effects[13]. Nevertheless, it is not clear *in vitro* and *in vivo* how high concentrations of glucose affect the production of reactive oxygen species by macrophages/monocytes and how statins affect their production.

Our aim in this study is to clarify in the J-774 macrophage-like cell line (the origin is mouse macrophages) how high glucose by itself and the addition of statins to high glucose affect the production of superoxide anion (O_2^-), the initial formation of reactive oxygen species. Furthermore, we estimated how the diabetic state affects O_2^- generation by peripheral blood monocytes in diabetic patients. To measure the O_2^- production, we employed a *Cypridina* luciferin analog (CLA) as an agent to amplify the luminescence, because *Cypridina* luciferin analog-dependent chemiluminescence appears to be highly dependent on the generation of superoxide anion[14].

MATERIALS AND METHODS

Materials

Diphenyleneiodonium chloride (DPI), GF-109203X (GF), and 12-O-tetradecanoylphorbol 13-acetate (TPA), geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP) were obtained from Sigma-Aldrich Japan. Mevalonic acid lactone (Sigma-Aldrich Japan) was converted to sodium mevalonic acid[15]. *Cypridina* luciferin analog was from Tokyo Kasei Kogyo (Tokyo, Japan). Mono-poly resolving medium was from Dainippon Pharmaceutical Co. (Tokyo, Japan). Cerivastatin was generously provided by Takeda Pharmaceutical Co. (Osaka, Japan), fluvastatin by Novaritis (Tokyo, Japan), and nisvastatin by Kowa Pharmaceutical Co. (Tokyo, Japan).

Subjects and isolation of peripheral blood monocytes

Subjects were nineteen type 2 diabetic outpatients (14 males and 5 females) and seventeen sex-and age-matched normal healthy volunteers (13 males and 4 females). Initially, diabetic patients were recruited on the basis of the following criteria : no proliferative retinopathy, no active impaired renal function (defined as a plasma creatinine >120 μmol/L), and no ketonuria. All subjects gave their informed consent to this study, and no subjects were taking any drugs known to affect monocyte functions. None of the subjects had an infectious disease, and the C-reactive protein level was not detectable. The mean age, BMI and HbA1c in the diabetic group were 48.5 ± 3.9 (mean ± SEM) years, 24.9 ± 0.9 kg/m^2, 9.4 ± 0.3 %, respectively. Monocytes were isolated from venous blood by mono − poly resolving medium and suspended in Hanks balanced salt solution (1×10^4 monocytes/ml).

J-774 cells culture with graded doses of glucose

J-774 cells were incubated with graded doses of glucose (5.6-33.3 mmol/L) in DMEM containing 10% FBS under an atmosphere of 5% CO_2 at 37 ℃ for 5 min, 8 h, 1 day, 2 days, and 7 days.

Effects of diphenyleneinodonium chloride and GF-109203X

To clarify whether the generated O_2^- is dependent on NADPH oxidase or protein kinase C, cells were pretreated with 20 μmol/L DPI (NADPH oxidase inhibitor) or 2 μmol/L GF (protein kinase C inhibitor) for 30 min just before the chemiluminescence assay.

Effects of statins with or without mevalonic acid and its metabolites

After 2-day incubation with graded doses (0-10 μmol/L) of statins (cerivastatin, fluvastatin, and nisvastatin) under high glucose (33.3 mmol/L) conditions, the suspension was centrifuged 5 min at room temperature at 250 x g to obtain cell fractions. The cells were sus-

pended in Hanks' balanced salt solution (1×10^6 cells/ml) for the assay, because DMEM is known to affect the assay. To test the effect of mevalonic acid and the metabolites, graded doses of mavalonic acids (1-100 μmol/L) or the metabolites (100 μmol/L GGPP or 100 μmol/L FPP) were added in the medium containing 10 μmol/L statins.

Measurement of superoxide anion generation

Chemiluminescence was measured with a Luminescence Reader (Model BLP 201, Aloka, Tokyo, Japan), as described previously[16]. The standard reaction mixture contained 9×10^5 cells, 5 μmol *Cypridina* luciferin analog, and 100 nmol/L TPA in Hanks' balanced salt solution in a total volume of 1 ml. All components, except for TPA, were preincubated for 3 min ; the reaction was initiated by the addition of TPA. Basal CL was assessed with the chemiluminescence value (KC/min/10^6 cells) just before the addition of TPA, and TPA-induced chemiluminescence (TPA-CL) was assessed with the maximally changed chemiluminescence value after TPA stimulation as KC/min/10^6 cells. All the reagents (DPI, GF, mevalonic acid, GGPP, and FPP) by themselves did not affect chemiluminescence in a cell free system (data not shown). The viability of cells measured by trypan blue staining was >97% at the beginning and end of the assay.

Statistical analysis

Statistical analysis was made with Student's unpaired *t* test. Results are expressed as the mean ± SEM of n observations. A value of $P < 0.05$ was considered to be statically significant. Experiments were repeated with essentially identical results.

RESULTS

Basal CL and TPA-CL in normal healthy subjects and diabetic patients (Fig. 1)

In diabetic patients, both basal CL and TPA-CL were significantly higher that those in control subjects.

Effect of high glucose on basal CL and TPA-CL (Figs. 2 and 3)

In the normal glucose condition (5.6 mmol/L), preincubation of J-774 cells with *Cypridina* luciferin analog resulted in an increase in chemiluminescence, which reached a plateau after 3 min (basal CL), and TPA resulted in a prominent increase in chemiluminescence, which reached a maximum after 10-16 min (TPA-CL). More than 2 days incubation of J-774 cells with 16.7 or 33.3 mmol/L glucose apparently increased both basal CL and TPA-CL compared to 5.6 mmol/L glucose. These effects were both dose and time dependent, except for 33.3 mmol/l TPA-CL at 5 min and 8 h. The 33.3 mmol/L glucose inhibited TPA-CL at 5 min and 8 h. These inhibitions may be partially dependent on the changes in cell volume due to high glucose-induced medium hyperosmolarity, because we have previously found in neutrophils that medium hyperosmolarity inhibited O_2^- generation due to cell shrinkage[17].

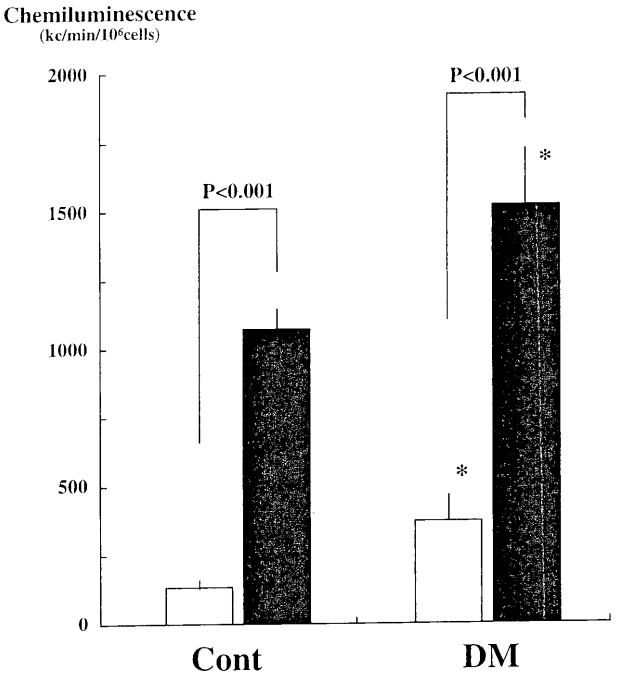

Fig. 1 Comparison of basal CL and TPA-CL between control and diabetic groups. Basal CL and TPA-CL by monocytes from seventeen normal healthy volunteers (Cont) and nineteen diabetic patients (DM) were measured as described in Methods. Open and closed bars indicate basal CL and TPA-CL, respectively. * $P < 0.05$ vs. same state in control subjects (Student's *t*-test).

Effect of DPI and GF on basal CL and TPA-CL (Figs. 4 and 5)

In Fig. 4, 20 μM DPI apparently diminished both basal CL and TPA-CL at both 5.6 and 33.3 mmol/L glucose. GF also inhibited both basal CL and TPA-CL at 33.3

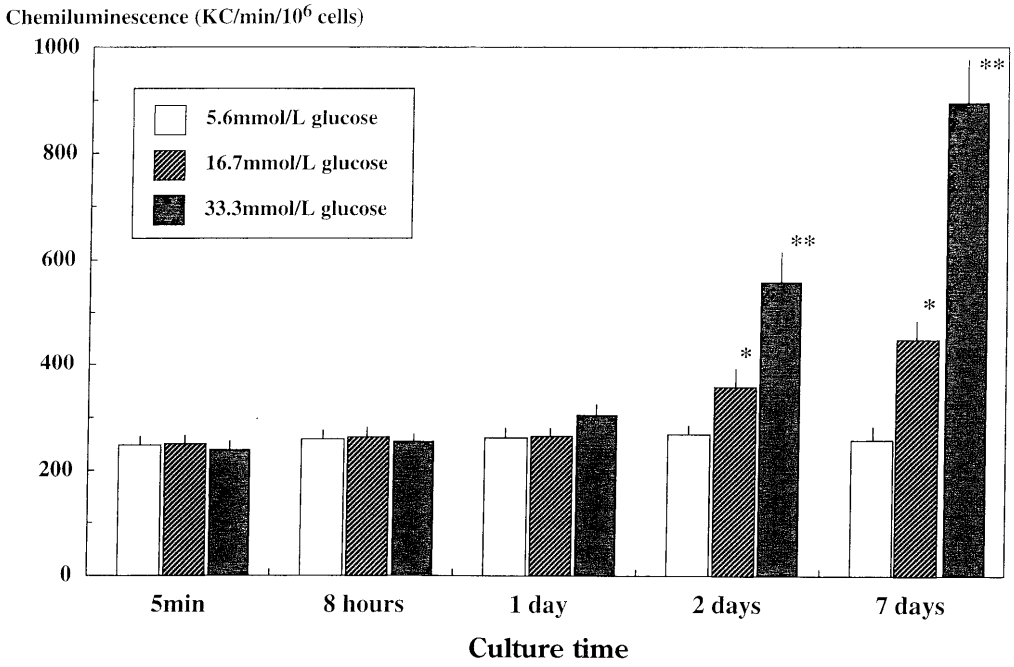

Fig. 2 Concentration-and time-dependent effects of glucose on basal O_2^- generation by J-774 cells. After cells were incubated with normal (5.6 mmol/L) or high (16.7 and 33.3 mmol/L) glucose for 5 min to 7 days, the basal O_2^- generation from cells was measured by chemiluminescence amplified with a Cypridina luciferin analog as described in Materials and Methods. $^*P < 0.05$, $^{**}P < 0.01$ vs 5.6 mmol/L in the observation time (N = 5 for all groups).

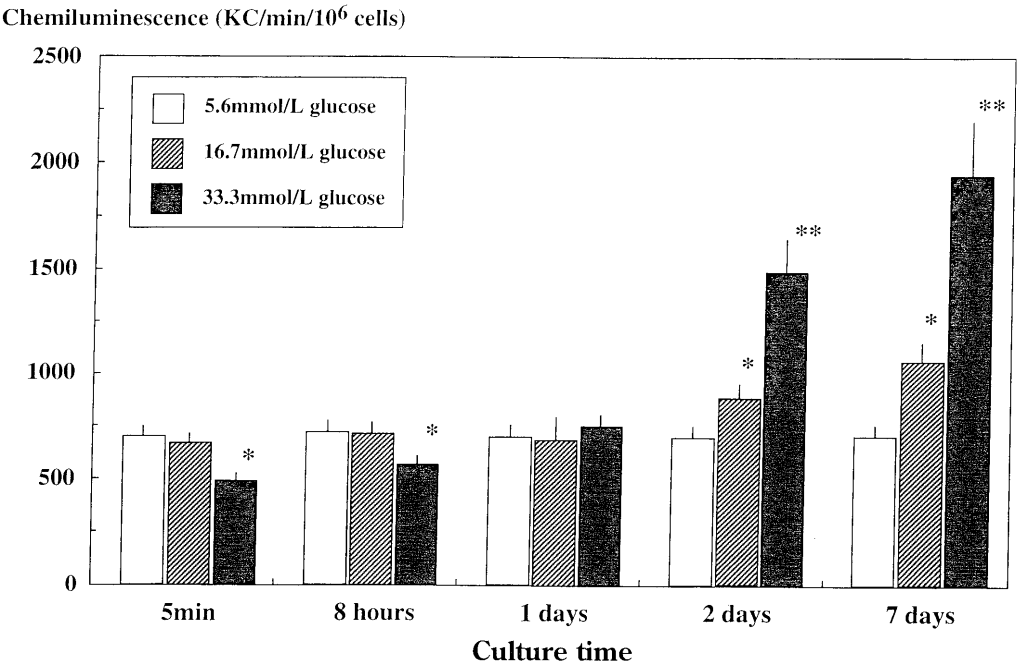

Fig. 3 Concentration-and time-dependent effects of glucose on TPA-induced O_2^- generation by J-774 cells. After cells were incubated with normal (5.6 mmol/L) or high (16.7 and 33.3 mmol/L) glucose for 5 min to 7 days, TPA-induced O_2^- generation by cells was measured as described in Materials and Methods. $^*P < 0.05$, $^{**}P < 0.01$ vs 5.6 mmol/L in the observation time (N = 5 for all groups).

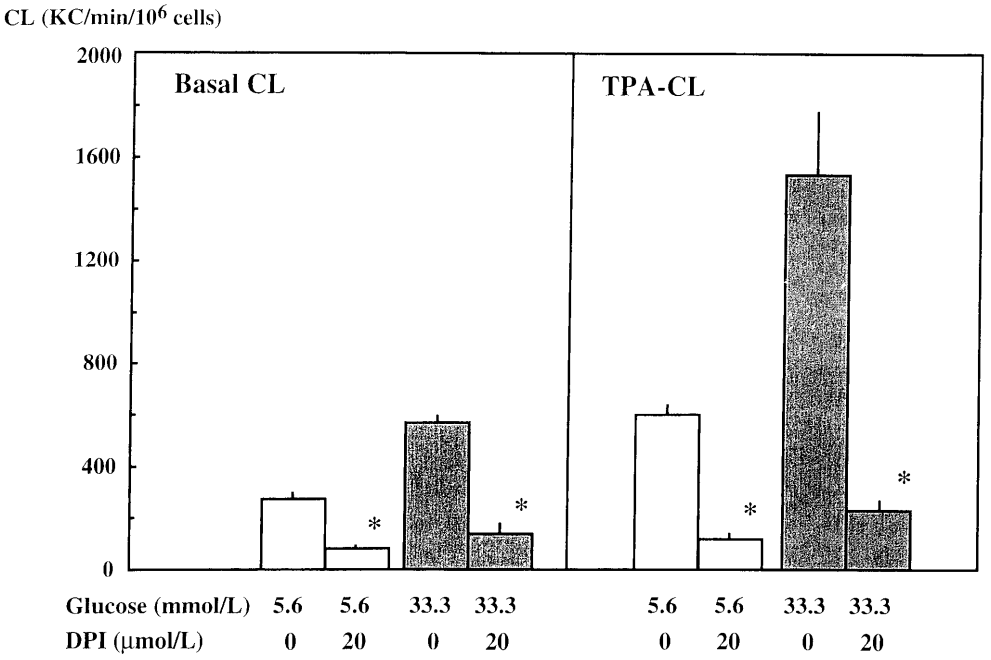

Fig. 4 Effect of diphenyleneiodonium chloride (DPI), a NADPH oxidase inhibitor, on basal and TPA-induced O_2^- generation under 5.6 and 33.3 mmol/L glucose conditions. Basal CL and TPA-CL indicate basal O_2^- generation and TPA-induced O_2^- generation, respectively. * $P < 0.001$ vs 0 μmol/L DPI in the same glucose concentration (N = 6 for all groups).

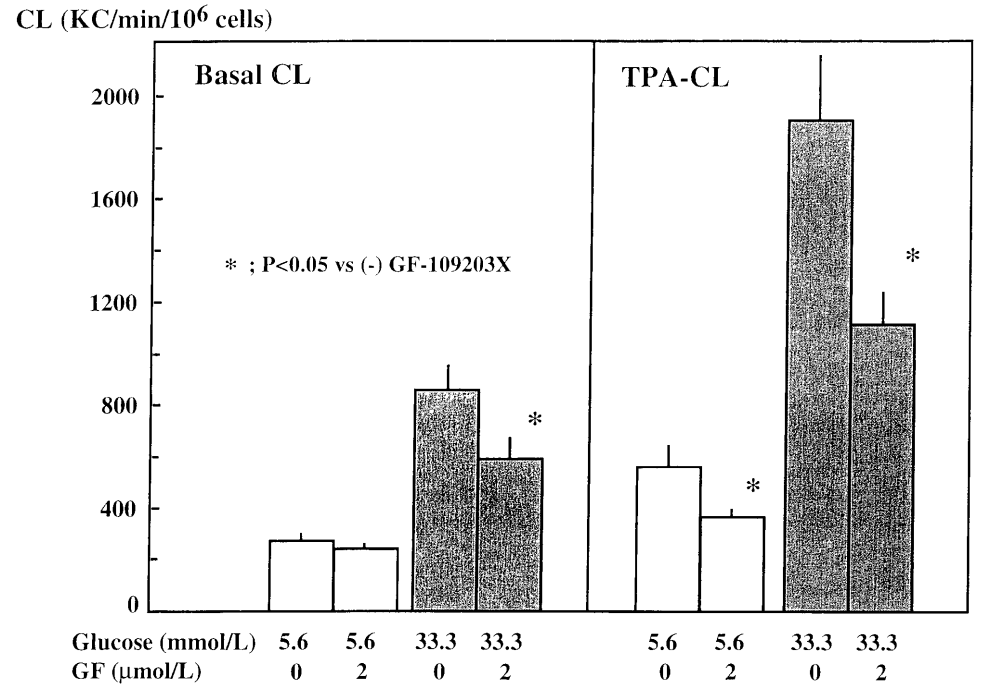

Fig. 5 Effect of GF-109203X (GF), a protein C kinase inhibitor, on basal and TPA-induced O_2^- generation under 5.6 and 33.3 mmol/L glucose conditions. Basal CL and TPA-CL indicate basal O_2^- generation and TPA-induced O_2^- generation, respectively. * $P < 0.05$ vs 0 μmol/L GF in the same glucose concentration (N = 6 for all groups).

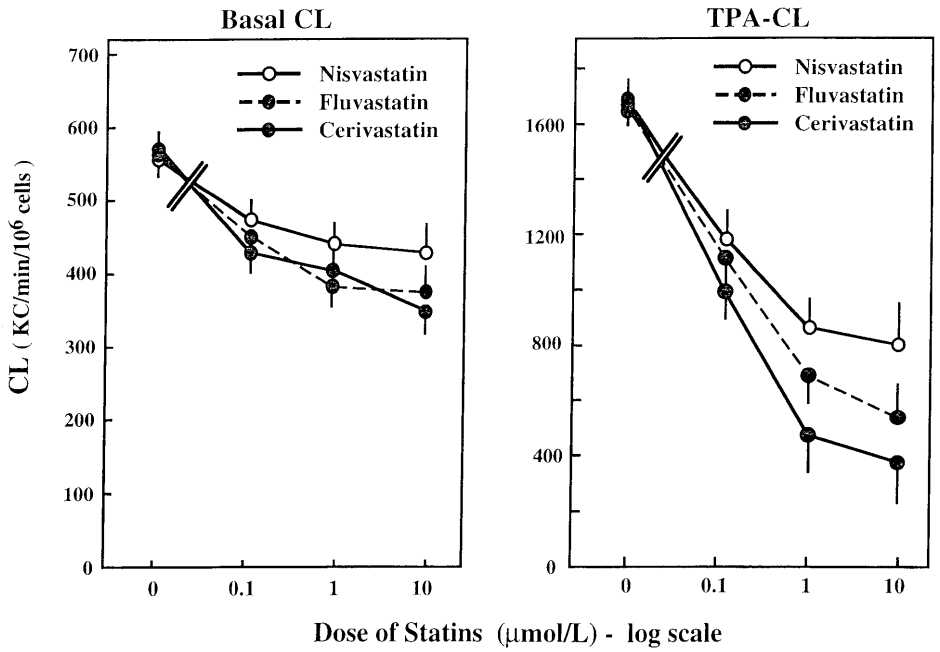

Fig. 6 Dose effect of various statins on high glucose-induced excessive O_2^- generation. Basal CL and TPA-CL indicate basal O_2^- generation and TPA-induced O_2^- generation, respectively. Cells were incubated with each of three statins (cerivastatin, fluvastatin, and nisvastatin) for 2 days under the 33.3 mmol/L glucose condition (N = 4 for all groups).

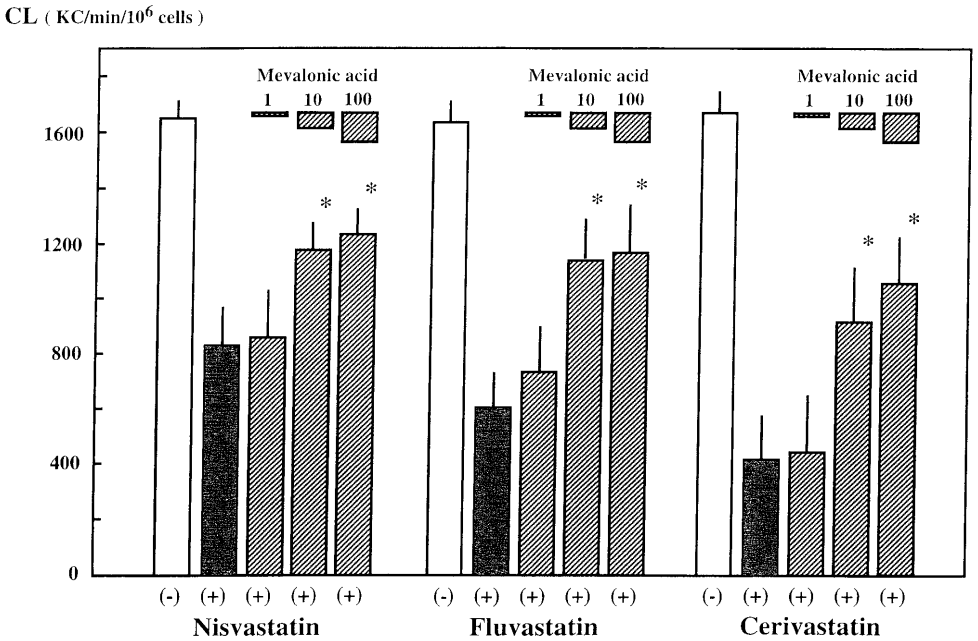

Fig. 7 Dose effect of mevalonic acid on statins' effect in TPA-CL by J-774 cells. Two days after co-incubation with various doses (0, 1, 10, and 100 μmol/L) of mevalonic acid and 10 μmol/L statin (cerivastatin, fluvastatin, or nisvastatin), TPA-induced O_2^- generation was measured as described in Material and Methods. * $P < 0.05$ vs statin alone (N = 6 for all groups).

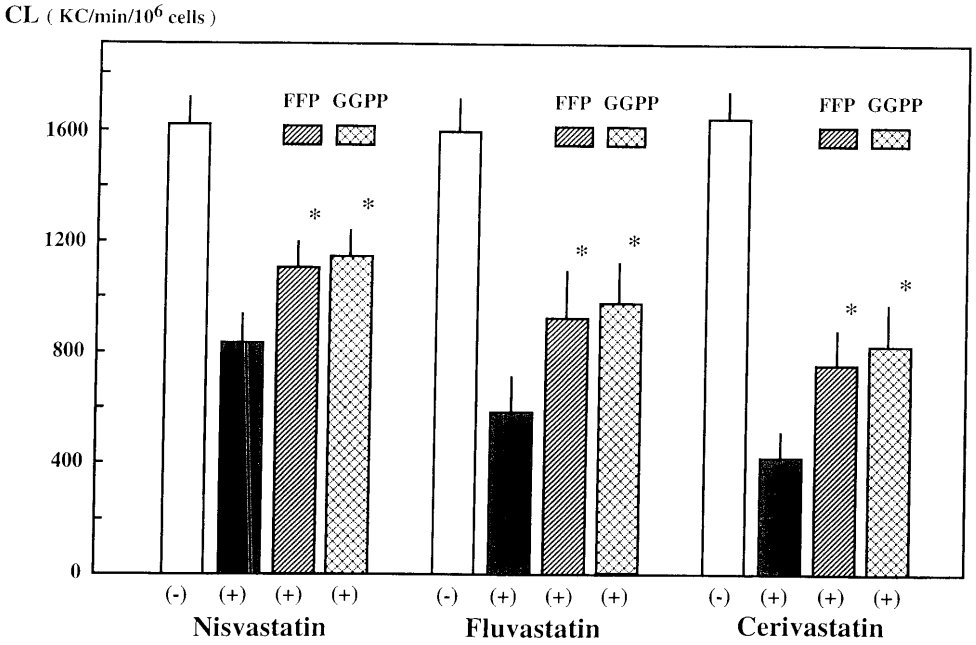

Fig. 8 Effects of the metabolites of mevalonic acid, geranylgeranyl pyrophosphate (GGPP) or farnesyl pyrophosphate (FPP), on statins' effect in TPA-CL by J-774 cells. Two days after co-incubation with 10 μmo/L statin (cerivastatin, fluvastatin, or nisvastatin) and 100 μmol/L mevalonic acid metabolite (GGPP or FPP), TPA-induced O_2^- generation was measured as described in Material and Methods. $^*P < 0.05$ vs statin alone (N = 6 for all groups).

mmol/L and TPA-CL at 5.6 mmol/L, whereas it did not affect basal CL at 5.6 mmol/L.

Effect of various statins on high glucose-induced basal CL and TPA-CL (Fig. 6)

Incubation with each of three statins (cerivastatin, fluvastatin and nisvastatin) for 24 h significantly inhibited high dose glucose (33.3 mmol/L)-induced increases in both basal and TPA-CL in a dose dependent manner. The serum max concentration of statins is almost 0.1 μmol/L for clinical treatment and in this *in vitro* study 0.1 μmol/L statins clearly inhibited high dose glucose-induced increases in both basal CL and TPA-CL.

Effect of mevalonic acid or its metabolites (GGPP and FPP) on statin-induced inhibition of TPA-CL at high dose glucose (Fig. 7 and 8)

Co-incubation with 10 and 100 μmol/L mevalonic acid clearly prevented a statin-induced decrease in TPA-CL (Fig. 7). Furthermore, 100 μmol/L GGPP and 100 μmol/L FPP also significantly prevented the stain-induced inhibition of TPA (Fig. 8).

DISCUSSION

Although glucose-induced oxidative stress is an attractive hypothesis for the pathogenesis of diabetic complications including atherosclerotic vascular disease[18], very little work has been published concerning the ability of peripheral blood monocytes and cultured macrophages to produce oxygen-derived free radicals under the diabetic state (high glucose conditions). In this study, basal and TPA-induced O_2^- productions in peripheral monocytes from diabetic patients were significantly increased. Furthermore, our current study clearly demonstrates that the culture of J-774 cells in a high glucose condition causes excessive O_2^- production. Thus, high glucose conditions lead to oxidative stress, which is due to excessive O_2^- production by monocytes *in vivo* and cultured macrophages *in vitro*. It is well known that monocyte/macrophage NADPH oxidase is a major source of O_2^- and that protein kinase C modifies NADPH oxidase[19]. In this study, DPI (NADPH oxidase inhibitor) and GF (protein kinase C inhibitor) clearly inhibited the high glucose-induced excessive O_2^- production by J-774 cells so that

the excessive O_2^- production must be due to an activation of NADPH oxidase by increasing protein kinase C activity. Interestingly, a higher glucose level leads to an increase in total diacylglycerol and in the activity of the membranous pool of protein kinase C in cultured smooth muscle and endothelial cells[20]. Furthermore, protein kinase C activity is increased by the plasma glucose concentration in human monocytes *in vivo*[19].

Statins competitively inhibit HMG-CoA reductase, the key enzyme in cholesterol biosynthesis, which in turn leads to reduce cholesterol production. In addition, concentrations of mevalonic acid and the metabolites (isoprenoid intermediates) such as GGPP and FPP are also decreased[12, 21]. GGPP and FPP are important for the isoprenylation of intracellular regulatory factors such as small G proteins (e.g., Rho A, Rac 1)[22, 23]. The pleiotropic effect of statins is thought to be, in part, dependent on inhibition of the synthesis of isoprenoid intermediates[13]. In this study, all three statins (cerivastatin, fluvastatin, and nisvastatin) clearly suppressed high glucose-induced excessive O_2^- production by J-774 cells and this *in vitro* inhibition by statins can be prevented partially by mevalonic acid and the metabolites GGPP and FPP. Therefore, the inhibition of high glucose-induced excessive O_2^- production by statins may be partially due to the inhibition of isoprenoid intermediate synthesis, and our data support the above concept of pleiotropic effects.

It was shown that high glucose-induced excessive O_2^- production in J-774 cells is related to NADPH oxidase and protein kinase C in this study, but it is not shown how statins affect the NADPH oxidase system. Wagner et al[15] reported that statins had no direct effect on either NADPH oxidase or protein kinase C activity and effectively lowered the NADPH oxidase-dependent O_2^- formation by preventing the isoprenylation of p21 Rac in endothelium-intact segments of rat aorta, although their experiments were performed under normal glucose conditions. Mevalonic acid and the metabolites could not completely prevent the statin' effect in this study and Weitz-Schmidt et al[24] demonstrated that, independent of the mevalonate pathway, satins can bind directly to lymphocyte functional antigen-1 and inhibit its interaction with ICAM-1. To elucidate in detail the mechanism by which statins affect high glucose-induced excessive O_2^- production in J-774 cells, further studies (e.g., their direct effects on NADPH oxidase and protein kinase C, isoprenylation of p21 Rac) would be required.

Enhanced oxidative stress has been observed in diabetic patients and it is thought that this enhanced oxidative stress is responsible for increased incidence of atherosclerosis, a major complication of diabetes mellitus. The present findings suggest that statins may be useful as a drug to prevent arteriosclerosis by inhibiting oxidative stress produced by macrophages in poorly controlled diabetic patients. Obviously, large-scale clinical intervention studies are needed to confirm a positive influence of statins on oxidative stress and rates of cardiac events in diabetic patients.

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