Glucocorticoid Excess Induces Superoxide Production in Vascular Endothelial Cells and Elicits Vascular Endothelial Dysfunction

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Abstract—Glucocorticoid (GC) excess often elicits serious adverse effects on the vascular system, such as hypertension and atherosclerosis, and effective prophylaxis for these complications is limited. We sought to reveal the mechanism underlying GC-induced vascular complications. Responses in forearm blood flow to reactive hyperemia in 20 GC-treated patients were significantly decreased to 43±8.9% (mean±SEM) from the values obtained before GC therapy (130±14%). An administration of vitamin C almost normalized blood flow responses. In human umbilical vein endothelial cells (HUVECs), production of hydrogen peroxide was increased up to 166.5±3.3% of control values by 10^-7 mol/L dexamethasone (DEX) treatment (P<0.01). Concomitant with DEX-induced hydrogen peroxide production, intracellular amounts of peroxynitrite significantly increased and those of nitric oxide (NO) decreased, respectively (P<0.01). Immunoblotting analysis using anti-nitrotyrosine antibody showed that peroxynitrite formation was increased in DEX-treated HUVECs. Using inhibitors against metabolic pathways for generation of reactive oxygen species (ROS), we identified that the major production sources of ROS by DEX treatment were mitochondrial electron transport chain, NAD(P)H oxidase, and xanthine oxidase. These findings suggest that GC excess causes overproduction of ROS and thereby perturbs NO availability in the vascular endothelium, leading to vascular complications in patients with GC excess. (Circ Res. 2003;92:81-87.)

Key Words: glucocorticoid  reactive oxygen species  nitric oxide  vascular endothelial function

Glucocorticoid (GC) has being used widely for the treatment of patients with various disorders including autoimmune diseases, allergic diseases, and lymphoproliferative disorders. It has been well known, however, that GC therapy using prednisolone, methylprednisolone, or dexamethasone (DEX) is often limited by several adverse reactions associated with GC excess.1,2 GC excess exhibits a variety of symptoms and signs, including truncal obesity with moon face, striae, hirsutism, cataract, osteoporosis, myopathy, diabetes mellitus, immunosuppression, and cardiovascular disorders such as hypertension and atherosclerosis.3 Among these, cardiovascular complications are one of the important factors for predicting the morbidity and mortality of patients with GC excess.3 Plasma volume expansion due to sodium retention plays a minor role,1,2,4 and increased peripheral vascular resistance due in part to an increased pressor response to catecholamines and angiotensin II is shown to play a major role in the pathogenesis of hypertension induced by GC excess.1,2,5 However, the molecular mechanism whereby GC excess causes the increase in vascular resistance remains unclear.

Vascular endothelial cells regulate vascular tone through the release of a variety of relaxing and contracting factors that modulate the contractile activity of vascular smooth muscle cells.6-7 Nitric oxide (NO), an endothelial cell-derived relaxing factor, is thought to be the most important vasoactive mediator for the reaction of endothelium-dependent vascular relaxation, and a decrease in its availability due to perturbation of synthesis and/or release of NO by vascular endothelial cells causes an increase in vascular resistance.6-8 Vascular endothelial cells are also capable of producing reactive oxygen species (ROS) such as superoxide, hydroxyl radicals, and hydrogen peroxide generated from various enzymatic sources. Because superoxide reacts with NO more promptly than with superoxide dismutase, intracellular overproduction of superoxide results in reduced NO availability through enhanced elimination of NO.3,9 Thus, we hypothesized that GC excess may elicit the overproduction of ROS from endothelial cells, thereby reduces NO availability to cause an impairment of relaxing activity of vascular smooth muscle cells.

The present study was undertaken to clarify whether GC excess affects endothelium-dependent vascular relaxation in
GC-treated patients and whether DEX treatment alters the production of hydrogen peroxide and the formation of peroxynitrite, a reactant molecule between NO and superoxide, in cultured human umbilical endothelial cells (HUVECs). The results demonstrate that GC excess potentially impairs endothelium-dependent vascular relaxation reaction in vivo and enhances ROS production to cause increased production of peroxynitrite in vitro.

Materials and Methods

Analysis of Responses in Forearm Blood Flow to Reactive Hyperemia and to Sublingual Administration of Nitroglycerin

Twenty patients (6 men and 14 women) aged 18 to 69 years (47 ± 3.6 years, mean ± SEM) treated with GC due to various autoimmune disorders were examined. They were treated with prednisolone, and ten of them were combined with pulse therapy using methylprednisolone. Total dose of GC corresponded to 3400 ± 692 mg of prednisolone. Among these, 5 patients were smokers and 2 patients were complicated with diabetes mellitus. Responses in forearm blood flow to reactive hyperemia and to sublingual administration of nitroglycerin (0.3 mg) were evaluated before and at 12 to 50 days (28 ± 2.4 days) after the start of GC therapy. Subjects were studied at the supine position in the morning after abstaining from food, alcohol, caffeine, and cigarettes for 12 hours. Medications were not changed during the study. Forearm blood flow was measured by venous occlusion method using strain-gauge plethysmography (mod- el ECSR, Hokanson, Inc) as reported elsewhere. The baseline value was measured after resting for 15 minutes, and then the upper arm was compressed by inflating pneumatic tourniquet to a pressure of 250 mm Hg for 5 minutes. The changes in forearm blood flow to reactive hyperemia were measured at 60 and 90 seconds after the cuff deflation. At least 15 minutes after the forearm blood flow returned to the basal level, 0.3 mg nitroglycerin was sublingually administered, and the changes in forearm blood flow were measured at 2, 3, 4, 5, and 6 minutes thereafter. The effects of vitamin C on the responses in forearm blood flow were also analyzed at 2 hours after a single oral administration of 2 g vitamin C during GC therapy. Maximum changes in forearm blood flow in response to both interventions were expressed as the percent changes from baseline values. Informed consent was obtained from each subject before the study. The protocol with regard to human subjects was followed according to the Declaration of Helsinki and the guidelines provided by the ethical review committee of Tokushima University Hospital.

Immunohistochemistry of Nitrotyrosine in Vascular Tissues

Vascular tissue samples of skeletal muscle obtained from three patients with GC excess, a 54-year-old male with Cushing syndrome associated with adrenal tumor (case 1), a 72-year-old female treated with GC for systemic lupus erythematoses (case 2), and a 40-year-old male treated with GC for polymyositis (case 3), were used. Case 1 had hypertension, and case 2 was a smoker. In case 1, rectus abdominal muscle was obtained at surgery for adrenal tumor, and in case 2 and 3, deltoid muscles were obtained by biopsy for the clinical diagnosis. We also examined rectus abdominal muscle obtained from a 55-year-old male with gastric cancer (control 1), a 46-year-old male (control 2), and a 50-year-old female (control 3) with myopathy as a control, because they had never received GC treatment. Control 3 was a smoker and had hypertension, but the other control cases did not have a smoking habit, hypertension, diabetes mellitus, or hyperlipidemia. All of the 6 cases had no established atherosclerosis, such as coronary artery diseases or cerebral vascular accident. Informed consent was obtained from each patient. The formation of peroxynitrite by GC excess in vivo was assessed using a mouse monoclonal antibody against nitrotyrosine (HyCult biotechnology b.v., Netherlands) as previously reported, and vascular endothelial cells were identified by staining with a rabbit polyclonal antibody against human von Willebrand factor (Sigma), followed by immunostaining with an ABC kit (Vector Laboratories Inc).

Cell Culture

HUVECs were obtained from Cell Systems Corp (Kirkland, Wash). They were cultured in collagen-coated dishes and maintained in CS-C serum-free medium under an atmosphere with 5% CO2 according to manufacturer’s instructions. Confluent cells after passages 3 to 6 were used in these experiments.

Detection of ROS Production by Electron Paramagnetic Response (EPR) Spin Trapping Method

ROS production in HUVECs treated with 10−7 mol/L DEX for 6 hours was evaluated by EPR spectroscopy using 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) (Sigma) for spin trapping. DMPO was used at a final concentration of 50 mol/L. EPR spectra were recorded by a JEOL FEIX spectrometer (JEOL USA) at room temperature with a microwave frequency of 9.785 GHz, a microwave power of 20 mW, a modulation frequency of 100 kHz, and a modulation amplitude of 0.5 G.

Analysis of Intracellular ROS, NO, and Peroxynitrite by Fluorescence Methods

The amounts of intracellular hydroxy peroxide, NO, and peroxynitrite produced in the presence of DEX (Sigma) were measured using intracellular fluorescent probes, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes) and diaminofluorescein-2 diacetate (DAF-2DA) (Dai-ichi Chemical Corp) and dihydrodihydroamine 123 (DHR) (Molecular Probe). Ten and 50 mol/L for DCFH-DA and DAF-2DA, respectively. In brief, after washing confluent HUVECs with Hanks’ balanced salt solution (HBSS) twice, cells were incubated at 37°C for 45 minutes with 20 μmol/L DCFH-DA for detecting hydrogen peroxide, at 37°C for 1 hour with both 10 mmol/L DAF-2 DA and 10 µmol/L acetylcysteine for detecting NO, and at 37°C for 1 hour with 50 mmol/L DHR for detecting peroxynitrite, respectively. After washing twice with HBSS, fluorescence intensities were measured by a spectrophotom- eter (Corona MTP-32) with excitation and emission wavelengths at 490 nm and 526 nm for hydroxy peroxide, 485 nm and 515 nm for DAF-2DA, and 480 nm and 530 nm for DHR, respectively. Changes in fluorescence intensities were expressed as percentages of the values obtained in the absence of DEX treatment.

Immunoblotting for Detecting Nitraed Tyrosine Residues of Proteins in HUVECs by DEX Treatment

Cell proteins were extracted from HUVECs treated with and without 10−7 mol/L DEX for 24 hours using cell lysis buffer (in mmol/L: 10 Tris-HCl, 150 NaCl, 5 EDTA, 1% NP-40) in the presence of protease inhibitor cocktail (Sigma). Twenty micrograms of proteins were electrophoresed in a 10% SDS-PAGE gel (Iwaki Glass) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked with 5% nonfat dry milk in TBS-T (20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 0.1% Tween-20, pH 7.6) followed by reacting with 0.4 μg/mL of anti-nitrotyrosine monoclonal antibody (HyCult biotechnology b.v.) for 1 hour. Membranes were then washed twice with TBS-T and incubated with horseradish peroxidase–conjugated sheep anti-mouse IgG (1:2000 dilution in TBS-T) for 1 hour. Immunocomplexes were detected by an enhanced horseradish peroxidase–luminol chemiluminescence reaction using ECL detection kit (Amersham Pharmacia Biotech, UK). The effects of superoxide dismutase (SOD) (200U/mL) (Sigma) and N-nitro-L-arginine methyl ester (L-NAME) (1 mmol/L) (Sigma), an inhibitor for NO synthase (NOS), on DEX-induced tyrosine nitration were also examined. As a positive control for nitration of tyrosine
residues, HUVECs were incubated with 1 mmol/L 3-morpholinosydnonimine (SIN-1) (Sigma), a donor of both NO and superoxide to increase peroxynitrite formation, for 1 hour. The intensities of bands were evaluated by densitometric analysis. The intensities of β-actin were used as an internal control for both protein application and transfer efficiency.

**Detection of Generation Sources of ROS in HUVECs by DEX Treatment**

To detect the generation sources of ROS in HUVECs by DEX treatment, the effects of various inhibitors for pathways involved in ROS generation were analyzed. After incubating HUVECs with 10^{-7} mol/L DEX for 24 hours, inhibitors were added to culture medium as following: indomethacin (100 μmol/L) (Sigma) for 15 minutes, L-NNAME (1 mmol/L) (Sigma) for 15 minutes, oxyurpinol (100 μmol/L) (Sigma) for 15 minutes, quinacrine (6 mmol/L) (Sigma) for 5 minutes, diphenyleneiodonium chloride (DPI) (50 μmol/L) (Alexis) for 5 minutes, and 4,4’-diisothiocyanato-stilbene-2,2’-disulfonic acid (DIDS) (100 μmol/L) (Sigma) for 10 minutes. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) (0.5 μmol/L) (Sigma) and thenoyltrifluoroacetone (TTFA) (100 μmol/L) (Sigma) were added with DEX, simultaneously. ROS assay were performed as described above.

**Statistical Analysis**

Data were expressed by mean±SEM and evaluated by one-way ANOVA to determine the statistical significance between groups. Multiple regression analysis was performed to analyze the relation between the effect of GC treatment on forearm blood response to reactive hyperemia and clinical parameters. Values of P<0.05 were considered to be statistically significant.

**Results**

**Responses in Forearm Blood Flow to Reactive Hyperemia and to Sublingual Administration of Nitroglycerin in Patients With GC Therapy**

Responses in forearm blood flow to reactive hyperemia in patients with GC therapy were significantly decreased to 43±8.9% from the values obtained before GC therapy (130±14%; n=20, P<0.01) (Figure 1A), whereas no effects on responses in forearm blood flow by nitroglycerin administration were detected (before 30±4.9% versus after 29±6.3%) (Figure 1A). Although serum total cholesterol was mildly elevated from 200±18 to 242±7.2 mg/dL (n=20, P<0.05) by GC therapy, the other parameters including blood pressure, body mass index, and serum triglyceride were not significantly altered by GC therapy. There was no significant change in fasting blood sugar level after GC therapy because the treatment for hyperglycemia was implemented in patients who developed diabetes mellitus by GC therapy. Multiple regression analysis showed that total dose of GC and period of GC therapy were significantly correlated with the decreases in forearm blood flow response to reactive hyperemia by GC therapy (P=0.045 and P=0.038, respectively). However, age, sex, the presence of coronary risk factors, the combination with pulse therapy, and other clinical parameters (basal levels of blood pressure, body mass index, serum triglyceride and total cholesterol, and the increase in total cholesterol after GC therapy) were not correlated with the decreases in forearm blood flow response to reactive hyperemia by GC therapy.

With a single oral administration of 2 g vitamin C, responses in forearm blood flow to reactive hyperemia in patients with GC therapy were remarkably improved from 43±8.9% to 122±14% (n=20, P<0.01) (Figure 1B). Responses in forearm blood flow to nitroglycerin administration were not affected with (30±5.6%) or without (29±6.3%) vitamin C (Figure 1B). These results indicate that GC therapy impairs the reaction of endothelium-dependent vasodilation and that the impaired reaction can be ameliorated by ROS scavenger.

**Immunostaining of Nitrotyrosine Residues in Vascular Tissues of Patients With GC Therapy**

Immunohistochemical study of vascular tissues obtained from skeletal muscles in three patients with GC excess (cases 1, 2, and 3) demonstrated that intimal regions of vascular walls identified by the staining pattern of von Willebrand factor were intensively immunostained with anti-nitrotyrosine antibody (Figure 2). Immunostaining patterns for nitrotyrosine residues in 41 among 59 arteries in case 1, 22 among 24 arteries in case 2, and 18 among 24 arteries in case 3 were similar to those shown in Figure 2. No appreciable immunoreactivity against nitrotyrosine residues was observed in 45 arteries and 17 arteries examined in control 1 and 2, respectively (Figure 2). Two among 26 arteries examined in control 3 were immunostained for nitrotyrosine residues. Because nitrotyrosine residues were formed mostly by the reaction of tissue proteins with peroxynitrite, a reaction product of NO and superoxide, these results are consistent.
with the assumption that there is an enhanced production of ROS with resultant increase in peroxynitrite at vascular walls in patients with GC excess.

**Effects of DEX Treatment on ROS Production in HUVECs**

In order to examine whether GC excess in fact enhances ROS production in vascular endothelial cells, ROS production was analyzed in DEX-treated HUVECs using EPR spectroscopy. As shown in Figure 3A, spectra showing hydroxyl radical signals were detected by incubation of HUVECs with 10^{-7} mol/L DEX for 6 hours. The signal amplitude in DEX-treated HUVECs was 8.4-fold compared with that of control. These signals were almost abolished by the treatment of SOD (200 U/mL).

We then analyzed the production of ROS, such as hydrogen peroxide, by DEX treatment in HUVECs using the fluorescence method. As shown in Figure 3B, the amounts of hydrogen peroxide produced were increased in a dose-dependent manner at 10^{-9}, 10^{-8}, and 10^{-7} mol/L DEX to 107.3 ± 7.6%, 154.5 ± 5.6%, and 166.5 ± 3.3% of the control values, respectively (n=24, P<0.01). Catalase (100 U/mL, Sigma), a scavenger for hydrogen peroxide, almost completely scavenged intracellular hydrogen peroxide produced by 10^{-7} mol/L DEX (Figure 3B). Furthermore, RU486 (10^{-5} mol/L, Sigma), a GC receptor antagonist, completely blocked the effect of DEX on the production of hydrogen peroxide (Figure 3B), indicating that DEX-induced ROS overproduction in HUVECs is mediated via GC receptor.

**Intracellular Changes of Hydrogen Peroxide, Peroxynitrite, and NO by DEX Treatment in HUVECs**

Because superoxide can react promptly with NO, it is plausible to assume that the overproduction of ROS by DEX elicits both a decrease in the level of NO and an increase in the formation of peroxynitrite in HUVECs. To test this possibility, intracellular amounts of hydrogen peroxide, peroxynitrite, and NO were measured before and at 6, 12, and 24 hours after DEX treatment in HUVECs. As shown in Figure 4A, production of hydrogen peroxide by 10^{-7} mol/L DEX significantly increased with time and reached to 132.5 ± 2.5% (n=24, P<0.01) of the baseline values, respectively, after 24 hours. Concomitant with this change, intracellular amounts of peroxynitrite significantly increased and those of NO decreased with a similar time course to 188.9 ± 2.7% (n=24, P<0.01) and 59.7 ± 0.58% (n=24, P<0.01) of the baseline values, respectively, after 24 hours.

**Enhanced Nitration of Tyrosine Residues of Proteins in HUVECs by DEX Treatment**

Immunoblotting analysis using an antibody against nitrotyrosine residues demonstrated that cell proteins extracted from...
Discussion

The present study demonstrated that responses in forearm blood flow to reactive hyperemia are drastically impaired by GC therapy in association with total dose of GC and period of GC therapy, and that ROS scavengers can ameliorate the impaired responses. Tagawa et al.\(^1\) showed that reperfusion after ischemia causes a release of NO from vascular endothelial cells, which leads to the relaxation of vascular smooth muscle cells. This reaction termed as endothelium-dependent vasodilatation is the mechanism of the vasodilatory responses of reactive hyperemia.\(^10\,11\) Therefore, the impairment of...
responses in forearm blood flow to reactive hyperemia in patients with GC therapy suggests reduced production and/or enhanced consumption of NO in the vascular endothelial cells. It was reported that reduced NO production caused by a downregulation of endothelial-inducible NOS contributes to the development of hypertension by GC excess.\(^23,24\) However, the present study demonstrated that the reduced responses in forearm blood flow to reactive hyperemia by GC therapy were ameliorated by the administration of antioxidant vitamin C, suggesting a potential role of ROS overproduction in patients with GC excess.\(^13\) Among ROS, superoxide does promptly react with NO, leading to the formation of peroxynitrite, which in turn nitrates tyrosine residues of proteins.\(^7,14\) Thus, the presence of nitrotyrosine residues is a biomarker showing the interaction between NO and superoxide.\(^7,14\) Our immunohistochemical study of vascular tissues in patients with GC excess clearly showed an increased immunostaining of nitrotyrosine at intimal regions as compared with those in patients without GC excess. Collectively, these results are consistent with the assumption that the impairment of the responses in forearm blood flow to reactive hyperemia in patients with GC therapy is primarily elicited by GC-induced ROS overproduction.

In spontaneously hypertensive rat, Suzuki et al\(^25\) reported that both serum levels of GC and oxidative stress in vascular endothelium were increased, and that the increased oxidative stress was suppressed by administration of a GC receptor antagonist. However, to our knowledge, no reports have been published concerning the effects of GC on ROS production in human vascular endothelial cells. The present study using cultured HUVECs revealed that GC enhances ROS production in HUVECs in a time- and dose-dependent manners. Additionally, the time-dependent overproduction of ROS by DEX treatment was associated with the time-dependent decline and increase of NO and peroxynitrite, respectively. Because peroxynitrite can nitrate tyrosine residues of intracellular proteins, the increased peroxynitrite formation by GC treatment was also confirmed by the increased nitrotyrosine residues in DEX-treated HUVECs. Thus, these in vitro findings are consistent with the data obtained by our clinical study and suggest that GC-induced ROS overproduction enhances the consumption of NO, resulting in the perturbation of NO availability and endothelium-dependent vasodilatory responses.

In vascular endothelial cells, ROS is known to be generated from several enzymatic sources, such as xanthine oxidase, NAD(P)H oxidase, NOS, and mitochondrial electron transport.\(^7\) It has been demonstrated that hypercholesterolemia enhances superoxide production through the activation of xanthine oxidase,\(^25,26,27\) and that native low-density lipoprotein increases superoxide generation from NOS.\(^28\) Other reports showed that thrombin stimulates superoxide production from NADPH oxidase in cultured human endothelial cells,\(^29\) and that NADH oxidase is a major source of superoxide anion in bovine coronary artery endothelium.\(^30\) Nishikawa et al\(^31\) reported that hyperglycemia increases superoxide production from mitochondrial electron transport chain in cultured bovine aortic endothelial cells. Our study using several inhibitors demonstrated that oxypurinol, quinacrine, DPI, DIDS, CCCP, and TTFA inhibited DEX-induced ROS production to almost the same extent. Based on these findings, we can consider that mitochondrial electron transport chain, xanthine oxidase, and NAD(P)H oxidase are major sources of GC-induced ROS production in HUVECs.

We showed that DEX-induced ROS overproduction was efficiently suppressed by an uncoupling agent of oxidative phosphorylation that can abolish mitochondrial transmembrane potential, as well as by inhibitors of complex-I and -II. These results suggest that GC-induced ROS overproduction is mainly derived from proton electrochemical gradient generated in mitochondrial electron transport chain. GC has been shown to stimulate oxidative phosphorylation in mitochondrial DNA (mtDNA) and to increase mitochondrial respiratory chain activity and mitochondrial RNA levels.\(^32,33\) It has also been reported that there were 6 sequence regions similar to genomic GC responsive element (GRE) in mitochondrial DNA (mtDNA) and that mtDNA is the primary site of action of steroid hormones.\(^32,33,35\) These observations suggest the presence of relationship between the action of GC on the transcription of mtDNA and the GC-induced ROS production. Interestingly, Marumo et al\(^36\) reported that GC inhibited superoxide production in a GC receptor–dependent way through the decrease in mRNA expression of p22phox, one of the components of NADPH oxidase, in human aortic smooth muscle cells. The actions of GC in vascular endothelial cells may be different from those in vascular smooth muscle cells. We also demonstrated that a GC receptor antagonist, RU486, completely blocked the GC-induced ROS overproduction. These results demonstrate that the action of GC on ROS production is mediated through GC receptors. Further studies should be needed to clarify the molecular mechanisms whereby GC affects the production sources of ROS via a GC receptor in vascular endothelial cells.

In the present study, we examined the acute effects of GC on the function and the ROS production in vascular endothelial cells both in vivo and in vitro. During the observed period, there were no vascular events, including hypertension and atherosclerotic diseases, in patients treated with GC, despite the appearance of vascular endothelial dysfunction. These findings suggest that the functional failure in compensatory mechanisms against scavenging the oxidant stress and other secondary events followed by ROS overproduction (ie, vascular remodeling associated with smooth muscle proliferation) may also be involved in the development of vascular complication in patients with GC excess. To clarify whether the ROS overproduction in vascular endothelial cells in acute phase of GC therapy is in fact related to GC-induced vascular complications in chronic phase, we should prospectively examine the relationship between GC-induced vascular en-
endothelial dysfunction and the development of vascular complications, and should analyze the effect of antioxidant treatment on the prevention of GC-induced vascular complications.

In summary, GC excess causes the overproduction of ROS and perturbation of NO availability in the vascular endothelium. The GC-induced decrease in NO availability may elicit vascular endothelial dysfunction, leading to hypertension and atherosclerosis, which are major cardiovascular complications in patients with GC excess.

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