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Only Two-Week Smoking Cessation Improves Platelet Aggregability and Intraplatelet Redox Imbalance of Long-Term Smokers

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OBJECTIVES We investigated whether and how soon smoking cessation ameliorates the smoking-induced intracellular oxidative stress and platelet aggregability in long-term smokers.

BACKGROUND Smoking is a major risk factor of atherothrombosis. Smoking cessation reduces cardiac events. However, the underlying mechanisms of the beneficial effects remain to be elucidated.

METHODS Twenty-seven male long-term smokers were divided into two groups. Group A (n = 14) quit smoking for four weeks whereas group B (n = 13) resumed smoking two weeks after quitting. Smoking status was monitored by measurement of urinary cotinine. Using gel-filtered platelets, agonist (adenosine diphosphate and collagen)-induced platelet aggregation, platelet-derived nitric oxide (PDNO), intraplatelet nitrotyrosine production, intraplatelet levels of the reduced form of glutathione (GSH) and its oxidized form (GSSG), and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and urinary 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), as markers of systemic oxidative stress, were measured. The baseline measurements were similar between the two groups.

RESULTS Smoking cessation quickly reduced agonist-induced platelet aggregations, intraplatelet nitrotyrosine level, and urinary productions of 8-OHdG and 8-iso-$PGF_{2\alpha}$ by two weeks in both groups. In group A, they were maintained at the low levels until four weeks, whereas they were reversed by resmoking in group B; PDNO release and intraplatelet GSH/GSSG ratio were time-dependently increased by smoking cessation but reversed by resmoking.

CONCLUSIONS The present findings are the first demonstration that only two weeks of smoking cessation can ameliorate the enhanced platelet aggregability and intraplatelet redox imbalance in long-term smokers, possibly by decreasing oxidative stress. Our findings may strengthen the motivation for smokers to quit smoking. (J Am Coll Cardiol 2005;45:589–94) © 2005 by the American College of Cardiology Foundation

Epidemiological studies have demonstrated that chronic smoking is a major risk factor for the development of atherosclerosis and thrombosis (1–3). These overwhelming epidemiological data closely link long-term smoking to adverse cardiovascular effects. Indeed, previous studies have shown the enhanced platelet aggregability (4–6) and the alterations in the clotting cascade in long-term smokers, suggesting the exaggerated risk of coronary artery thrombosis (7,8).

Platelets possess the L-arginine–nitric oxide (NO) pathway through constitutive NO synthase in humans (9,10). We have previously shown that long-term smoking impairs platelet-derived nitric oxide (PDNO) release (6), which acts as a negative feedback mechanism to inhibit not only platelet aggregation (11) but also recruitment after aggregation (12). Furthermore, we have recently shown that impaired PDNO bioactivity and augmented platelet aggregability in long-term smokers are related to the imbalance of the intraplatelet redox state, suggesting the smoking-induced oxidative stress for platelet activation (5,6). Thus, smoking-induced platelet-mediated thrombotic mechanisms may be involved in the pathophysiology of coronary artery disease of long-term smokers. It has been previously reported that smoking cessation is associated with a reduction in the risk of cardiovascular disease (13,14). However, the underlying mechanisms of the beneficial effects remain unclear. Accordingly, we investigated whether and how soon smoking cessation ameliorates impaired PDNO bioactivity and augmented platelet aggregability by improving the imbalance of the intraplatelet redox state. To assess this issue, we examined the effects of smoking cessation on platelet aggregation, PDNO bioactivity, intraplatelet nitrotyrosine production, the intraplatelet redox state, and systemic oxidative stress.

METHODS

Study subjects. The study groups consisted of 27 healthy male medical students in our university who smoked at least 15 cigarettes per day for more than 5 years (mean age, 27.4 years old). They were randomly divided into the two groups after baseline measurements (Table 1). Group A (n = 14) quit smoking for 28 days, whereas group B (n = 13)

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Abbreviations and Acronyms

ADP	= adenosine diphosphate
GSH	= reduced form of glutathione
GSSG	= oxidized form of glutathione
NO	= nitric oxide
PDNO	= platelet-derived nitric oxide
PRP	= platelet-rich plasma
PPP	= platelet-poor plasma
8-iso-$PGF_{2\alpha}$	= 8-iso-prostaglandin $F_{2\alpha}$
8-OHdG	= 8-hydroxy-2′-deoxyguanosine

resumed smoking at day 14 after quitting. We told the volunteers at the beginning of the study not to change their lifestyle. Smokers had abstained from smoking for at least 120 min before initiating the protocol to avoid the acute effects of smoking on platelet function. Thereafter, blood sampling was performed at 7, 14, 21, and 28 days, and urinary sampling was performed at 14 and 28 days after the start of the protocol. Smoking status was monitored by weekly measurements of urinary cotinine in all subjects. The protocol was approved by the institutional ethics committee. Written informed consent was obtained from all subjects.

Preparation of washed platelets. Platelet suspensions were prepared as described previously (4). Briefly, blood (20 ml) was collected by venipuncture into a plastic tube containing 3.15% trisodium citrate. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared according to the previously described method (15). The platelets counts were adjusted to 2×10^5 platelets/μl in Tyrode's solution, the composition of which was described previously (4).

Measurements of platelet aggregation. We measured adenosine diphosphate (ADP)- and collagen-induced platelet aggregations as described previously (5,6). In brief, ADP (5 and 10 μmol/l) and collagen (0.2 and 0.5 μg/ml) were added to the washed platelet suspensions, and light transmission was monitored by using a platelet aggregometer (MDM Hematracer, MC Medical Co., Tokyo, Japan).

Table 1. Baseline Characteristics

	Group A (n = 14)	Group B (n = 13)
Age (yrs)	29 ± 8	27 ± 5
Gender (male/female) (n)	14/0	13/0
Body mass index (kg/m^2)	22 ± 2	22 ± 2
Cigarettes per days (n)	21 ± 7	23 ± 8
Smoking period (yrs)	10 ± 5	9 ± 4
Systolic/diastolic blood pressure (mm Hg)	118 ± 13	115 ± 14
Heart rate (beats/min)	71 ± 7	72 ± 9
Total cholesterol (mg/dl)	176 ± 30	175 ± 14
HDL cholesterol (mg/dl)	58 ± 10	59 ± 8
LDL cholesterol (mg/dl)	100 ± 19	99 ± 13
Fasting blood glucose (mg/dl)	88 ± 7	88 ± 9
Fibrinogen (mg/dl)	202 ± 28	291 ± 47
Plasma epinephrine (mg/dl)	38 ± 21	41 ± 20
Plasma norepinephrine (pg/dl)	313 ± 72	319 ± 85
Urinary cotinine (ng/ml)	1,121 ± 559	1,158 ± 695

Values are presented as mean ± SD. All p = NS.
HDL = high-density lipoprotein; LDL = low-density lipoprotein.

Measurements of PDNO. We measured NO by using an NO meter (Model N0-501, Inter Medical Co., Tokyo, Japan) as described previously (4). After the baseline electric was stabilized, an ADP-induced (50 μmol/l) electrical current was recorded at the rate of 20 mm/min, and a change in the peak electrical current was considered as an index of the NO release.

Measurements of intraplatelet redox status. We measured intraplatelet GSH (the reduced form of glutathione) and GSSG (the oxidized form of glutathione) by high-performance liquid chromatography (HPLC) with an electrochemical detection system (ECD-300, Eicom Co., Kyoto, Japan) as previously described (16). The analytical column was a 150 × 4.6 mm, SC-5 ODS (Eicom Co). For HPLC measurements, PRP and PPP were mixed with $HClO_4$ (final concentration, 6%), sonicated for 5 s with a tip sonicator (Model MS-50, Heat System-Ultrasonic Inc., Farmingdale, New York), and centrifuged at 12,000 *g* for 2 min. The supernatant was then stored at −80°C until injection into the HPLC column. Intraplatelet GSH/GSSG ratio was calculated as an index of intraplatelet oxidative stress.

Detection of intraplatelet nitrotyrosine. We measured intraplatelet nitrotyrosine production by using a modified method of a previous study. Immunolabeling was performed by using a polyclonal antibody to nitrotyrosine as a primary antibody and fluoresein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) as a secondary antibody, and then analyzed with the FACScan (Becton-Deckinson, San Diego, California). The results were expressed as the percent changes in nitrotyrosine-specific staining of platelets by collagen-induced platelet aggregation in each group.

Measurements of urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-iso-prostaglandin $PGF_{2\alpha}$ (8-iso-$PGF_{2\alpha}$). We measured urinary 8-OHdG, an indicator of DNA oxidation (ELISA kit, Japan Institute for the Control of Aging, Tokyo, Japan), and urinary 8-iso-$PGF_{2\alpha}$, a product of lipid peroxidation (Cayman Chemical Company, Ann Arbor, Michigan), using 24-h urine samples, according to previously described procedures (17–19).

Statistical analysis. Values are presented as means ± SD. Statistical comparisons between groups were performed by an unpaired Student *t* test. Multiple comparisons were analyzed by two-way repeated-measures analysis of variance with a post-hoc Scheffé's test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Baseline characteristics of the study subjects. The body mass index distribution of the subjects was similar between the two groups (Table 1). All subjects had no other major risk factors including hypercholesterolemia, hypertension,