Long-Term Smoking Causes Nitroglycerin Resistance in Platelets by Depletion of Intraplatelet Glutathione

Nobuya Haramaki, Hisao Ikeda, Yoshinori Takajo, Atsushi Katoh, Seiji Kanaya, Satoshi Shintani, Reiko Haramaki, Toyoaki Murohara, Tsutomu Imaizumi

Abstract—We investigated whether platelet responsiveness to nitroglycerin (NTG) is maintained in long-term smokers and if not, the mechanism. In the absence or presence of NTG, intraplatelet reduced glutathione (GSH) levels and ADP-induced platelet aggregation and intraplatelet cGMP levels were measured in 10 long-term smokers and 10 age-matched nonsmokers. The intraplatelet GSH level was significantly lower in smokers than in nonsmokers ($P<0.05$). Platelet aggregation was dose-dependently inhibited by NTG in both groups; however, inhibition was significantly weaker in smokers. $\alpha$-Acetylcysteine (1 mmol/L), an exogenous thiol agent, significantly potentiated NTG-induced platelet inhibition in nonsmokers but not in smokers. The ADP-induced intraplatelet cGMP level was significantly greater in the presence of NTG in nonsmokers but not so in smokers. Because the effects of long-term smoking are multifactorial, a rabbit model was made by chronic administration of buthionine sulfoximine (BSO, $n=6$) to decrease intraplatelet GSH. The intraplatelet GSH level was significantly lower in BSO-treated rabbits than in saline-treated rabbits ($P<0.001$). The NTG-induced inhibition of platelet aggregation was significantly weaker in BSO rabbits. $\alpha$-Acetylcysteine–induced potentiation was not observed in BSO rabbits, whereas significant potentiation was found in saline rabbits. These findings were similar to those of long-term smokers. In contrast, the intraplatelet GSH–to–oxidized glutathione ratio, which represents the redox state of glutathione, was significantly lower in smokers than in nonsmokers, whereas no difference was found between saline rabbits and BSO rabbits. In conclusion, long-term smoking causes NTG resistance to aggregation in platelets, possibly through the depletion of intraplatelet GSH.

Keywords: smoking • nitroglycerin • platelets • glutathione • cGMP

Organic nitrates such as nitroglycerin (NTG) have been widely used to treat cardiovascular diseases, including ischemic heart disease and congestive heart failure. The expected clinical benefit of organic nitrates is mainly attributed to their vasodilator properties. However, organic nitrates are also known to possess antiplatelet effects, and several studies have indicated that the clinical actions of organic nitrates include inhibition of platelet aggregation. A poor hemodynamic response to organic nitrates has been observed in patients with congestive heart failure, and this phenomenon has been termed nitrate resistance. Nitrate resistance has also been observed in platelets from patients with hyperten-

sion, obese diabetes, and stable angina pectoris. However, the mechanism of nitrate resistance is still unknown. Previous studies have suggested that the antiplatelet effects of organic nitrates are mediated through their conversion (biotransformation) to nitric oxide (NO). NO inhibits both adhesion and aggregation of platelets by increasing the level of intraplatelet cGMP through the activation of soluble guanylate cyclase. In the process of biotransformation, sulphydryl-containing compounds, thiols (eg, cysteine and GSH, the reduced form of glutathione), are required. Thiol, especially intracellular GSH, are efficient antioxidants, and depletion of thiols in tissues under oxidative conditions has been reported. Therefore, it is speculated that the biotransformation of organic nitrates to NO could be disturbed under oxidative conditions. However, data regarding the potential role of intraplatelet GSH in the antiplatelet effects of organic nitrates under oxidative conditions are limited.

Oxidative stress, which is defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses, is enhanced in long-term smokers, and numerous epidemiological studies have established that cigarette smoking is a major risk factor for atherosclerosis. In addition, augmented platelet aggregability in habitual smokers has been reported. Therefore, platelet-mediated thrombotic mechanisms could be involved in the pathophysiology of ischemic heart disease observed in...
smokers. However, it is not known whether the effects of exogenous NO donors, such as organic nitrates, on platelet aggregation are maintained in smokers. In the present study, we examined the effects of NTG on platelet aggregation in long-term smokers and found that NTG resistance was associated with decreased GSH levels in the platelets of smokers. Because the effects of cigarette smoking on NTG resistance are multifactorial,23 we further explored the involvement of intraplatelet GSH to elucidate the mechanisms of NTG resistance by using rabbits treated with a specific GSH synthesis inhibitor, by which intraplatelet GSH was decreased to levels as low as those found in smokers.

## Methods

### Materials

All chemicals were purchased from Sigma Chemical Co. l-Butiophene-[S,R]-sulfoximine (BSO) is a specific inhibitor of $\gamma$-glutamylcysteine synthetase, a key enzyme for the rate-limiting step in GSH synthesis.24 $N$-acetylcysteine (NAC) is identical to $N$-acetylcysteine (NAC) except that the former contains a hydroxyl group in place of a sulfhydryl group.

### Study Subjects

The study groups consisted of 10 male smokers who had smoked at least 15 cigarettes per day for $\geq$5 years and 10 healthy, age-matched, male nonsmokers who had never smoked. None of the subjects presented evidence of other major risk factors for atherosclerosis, such as hypercholesterolemia, hypertension, or diabetes mellitus. Long-term smokers had abstained from smoking for at least 120 minutes before the start of the investigations to avoid the short-term effects of smoking on platelet function. This study was approved by our institutional Ethics Committee, and informed consent for the study was obtained from all subjects. As shown in Table 1, the 2 groups did not differ in terms of blood pressure, heart rate, total cholesterol, HDL cholesterol, LDL cholesterol, fasting plasma glucose, and platelet count. The plasma nicotine level was 10.9 $\pm$ 3.6 ng/mL in smokers but was not detectable in nonsmokers.

### Preparation of PRP and PPP and Ex Vivo Platelet Aggregation

Twenty milliliters of blood 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 a previously described method.25 The platelet concentration in PRP was adjusted to $3 \times 10^8$ platelets/μL by adding PPP and was used for the following examinations. ADP-induced platelet aggregation was measured. Experimental conditions (the presence of NTG, NAC, and NAS) for platelet aggregation are described in the figure legends. Platelet aggregation was initiated by the addition of ADP (1 to 10 μmol/L) and was monitored for 7 minutes by continuous recording of light transmission in an 8-channel platelet aggregometer (MDM Hematracer, MC Medical Co). The maximal extent of aggregation was expressed as the percent change in light transmission by considering the transmission through PPP as 100% and that of PRP as 0%.

### Plasma and Intraplatelet Concentrations of GSH

We measured plasma and intraplatelet GSH, the reduced form of glutathione, by high-performance liquid chromatography (HPLC) with an electrochemical detection system (ECD-300, Eicom Co) as previously described.26 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 seconds with a tip sonicator (model MS-50, Heat Systems-Ultrasonics Inc), and centrifuged at 12,000g for 2 minutes. The supernatant was then stored at $-80^\circ$C until injection into the HPLC column. The concentration of GSH in PRP was higher than that in PPP. Accordingly, the intraplatelet concentration was calculated from the difference between PRP and PPP because the difference was assumed to be due to the existence of platelets.

### Intraplatelet Redox Status of Glutathione

In nonsmokers, smokers, saline-treated rabbits, and BSO-treated rabbits (n = 6 each), PRP was prepared from whole EDTA-anticoagulated blood and immediately gel filtered to isolate platelets from plasma. The gel-filtered platelets were then deproteinized with HClO$_4$ as described above, and 10 μL of the sample was subjected to HPLC to measure GSH and GSSG (the oxidized form of glutathione). The HPLC postcolumn reaction with orthophthalaldehyde-hydrazide at a high pH was performed as previously described.27 The analytical column was a 150×3.0-mm SC-5 ODS (Eicom), and the fluorescence detector was an L-7485 (Hitachi Ltd).

### Measurements of Intraplatelet cGMP Levels

We measured intraplatelet cGMP levels after ADP-induced platelet aggregation by modifying the assay in our previous report.28 In brief, at the end of platelet aggregation experiments, the aggregated platelets were rapidly mixed with HClO$_4$ (final concentration, 6%). PRP without stimulation with ADP was also mixed with HClO$_4$ in the same manner. The mixed suspension was then sonicated twice for 5 seconds with a tip sonicator and centrifuged at 12,000g for 2 minutes. The supernatant was neutralized with 60% KOH. After centrifugation, cGMP in the samples was measured by a radioimmunoassay from a commercially available kit (Yamasu Shoyu Co). The changes in ADP-induced platelet cGMP levels were expressed

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**TABLE 1. Baseline Characteristics of Nonsmokers and Smokers**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonsmokers (n=10)</th>
<th>Smokers (n=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>33±3</td>
<td>33±3</td>
<td>NS</td>
</tr>
<tr>
<td>Male/female, n/n</td>
<td>10/0</td>
<td>10/0</td>
<td>NS</td>
</tr>
<tr>
<td>Cigarettes smoked per day</td>
<td>0</td>
<td>24±7</td>
<td></td>
</tr>
<tr>
<td>Smoking period, y</td>
<td>0</td>
<td>13±4</td>
<td></td>
</tr>
<tr>
<td>Systolic/diastolic blood pressures, mm Hg</td>
<td>122±12/69±9</td>
<td>124±14/70±8</td>
<td>NS</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>68±10</td>
<td>71±9</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>173±17</td>
<td>184±17</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>53±10</td>
<td>50±12</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>104±14</td>
<td>114±25</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting plasma glucose, mg/dL</td>
<td>91±8</td>
<td>85±10</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet count, 10$^4$/μL</td>
<td>23.7±1.8</td>
<td>22.5±2.2</td>
<td>NS</td>
</tr>
<tr>
<td>Nicotine, ng/mL</td>
<td>Not detectable</td>
<td>10.9±3.6</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD.
Saline-treated and BSO-treated rabbits

In long-term smokers, the NTG-induced increase in intraplatelet cGMP was significantly weaker than that in nonsmokers, suggesting NTG resistance in the platelets of long-term smokers. The intraplatelet concentration of GSH was significantly lower in long-term smokers. Platelets obtained from rabbits in which intraplatelet GSH was decreased by treatment with BSO exhibited NTG resistance as well.

**Treatment With BSO**

The animal investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1985) and was approved by the Animal Research Committee of Kurume University School of Medicine. Male New Zealand White rabbits (Kyudo, Fukuoka, Japan) weighing 2.5 to 3.0 kg were used in this study. The animals were randomly divided into 2 groups. Animals in the BSO group were given BSO (1 g IP) 3 times per 24 hours. Animals in the saline group received saline instead of BSO. Two hours after the last injection of BSO or saline, the rabbits were anesthetized with ketamine and xylazine, and then 20 mL of blood was collected by venipuncture into a plastic tube containing 3.15% trisodium citrate. Then PRP and PPP were prepared as described above.

**Statistical Analysis**

Data are expressed as mean±SD. Comparisons between the 2 groups were performed with a Student’s t test. Multiple comparisons were performed with ANOVA and the Bonferroni test. Differences were considered statistically significant when \( P<0.05 \).

**Results**

No difference was observed in the plasma concentrations of GSH between long-term smokers and nonsmokers. However, intraplatelet GSH was significantly lower in long-term smokers than in nonsmokers (Table 2, top). In both groups, platelet aggregation was stimulated by ADP (1.0 to 10.0 \( \mu \text{mol/L} \)) in a dose-dependent manner (data not shown). The aggregation tended to be greater in long-term smokers; however, it was not statistically significant. At 5.0 \( \mu \text{mol/L} \) ADP, the effect of NTG on platelet aggregation was tested. In both groups, platelet aggregation was dose-dependently inhibited by NTG; however, inhibition was significantly blunted in smokers (Figure 1). NAC (1 mmol/L) significantly potentiated NTG-induced inhibition in nonsmokers but not in smokers. Representative recordings of platelet aggregation are shown in Figure 1 (upper panel), and pooled data are also shown in Figure 1 (lower panel).

Intraplatelet cGMP levels after ADP-induced platelet aggregation are shown in Table 3. In nonsmokers, NTG increased the intraplatelet cGMP level in a dose-dependent manner, and it was further enhanced in the presence of NAC. In long-term smokers, the NTG-induced increase in intraplatelet cGMP was significantly weaker than that in nonsmokers. In addition, NAC did not significantly potentiate the increase in intraplatelet cGMP in long-term smokers. The plasma level of GSH in BSO-treated rabbits was not different from that in saline-treated rabbits (Table 2, bottom). However, the intraplatelet GSH in BSO-treated rabbits was significantly lower than that in saline-treated rabbits.

Figure 2 shows the effects of NAC and NAS on NTG-induced inhibition of platelet aggregation in the saline and BSO groups. In both groups, platelet aggregation was dose-dependently inhibited by NTG (5 to 50 \( \mu \text{g/mL} \)); however, inhibition was significantly blunted in the BSO group. NAC (1 mmol/L) significantly potentiated the NTG-induced inhibition in the saline group but not in the BSO group. NAS did not affect the NTG-induced inhibition of platelet aggregation in either group.

The intraplatelet glutathione redox state is shown in Table 4. Total glutathione and GSH were significantly lower in smokers and BSO rabbits than in nonsmokers and saline rabbits, respectively. The GSH-to-GSSG ratio was significantly lower in smokers than in nonsmokers; however, no difference in the GSH-to-GSSG ratio was observed between saline rabbits and BSO rabbits.

**Discussion**

In the current study, the antiaggregating and intraplatelet cGMP-stimulating effects of NTG were reduced in platelets obtained from long-term smokers compared with those from nonsmokers, suggesting NTG resistance in the platelets of long-term smokers. The intraplatelet concentration of GSH was significantly lower in long-term smokers. Platelets obtained from rabbits in which intraplatelet GSH was decreased by treatment with BSO exhibited NTG resistance as well. These results suggest that NTG resistance in long-term smokers may explain the reduced effectiveness of NTG in these patients.

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**TABLE 2. Plasma and Intraplatelet Concentrations of GSH**

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td><strong>Non-smokers and smokers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma GSH, ( \mu \text{mol/L} )</td>
<td>1.10±0.48</td>
<td>1.01±0.51</td>
</tr>
<tr>
<td>Intraplatelet GSH, mol/10(^{18}) platelets</td>
<td>10.25±4.90</td>
<td>6.51±1.40*</td>
</tr>
<tr>
<td><strong>Saline Group</strong></td>
<td><strong>BSO Group</strong></td>
<td></td>
</tr>
<tr>
<td>Plasma GSH, ( \mu \text{mol/L} )</td>
<td>1.17±0.27</td>
<td>1.15±0.31</td>
</tr>
<tr>
<td>Intraplatelet GSH, mol/10(^{18}) platelets</td>
<td>8.85±2.74</td>
<td>3.02±1.14*</td>
</tr>
</tbody>
</table>

All values are expressed as mean±SD.

*\( P<0.05 \) compared with nonsmokers.
†\( P<0.001 \) compared with saline group.

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**Figure 1.** Platelet aggregation in nonsmokers and smokers. Top, representative recordings. Platelet aggregation was induced by NTG (5 \( \mu \text{mol/L} \)) in the presence of 4 different doses of NTG (0, 10, 25, and 50 \( \mu \text{g/mL} \)) and NAC (1 mmol/L). Bottom, pooled data. Conditions were as follows: PRP obtained from nonsmokers (▫), PRP obtained from nonsmokers with 1 mmol/L NAC (▫), PRP obtained from smokers (●), and PRP obtained from smokers with 1 mmol/L NAC (▲). *\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \) for nonsmokers vs smokers. ††\( P<0.01 \) for the absence vs presence of NAC.
smokers is caused by decreased intraplatelet concentrations of GSH.

In long-term smokers, platelet aggregability in response to ADP tended to be enhanced, but it was not statistically significant. These results agree with those of our previous report.28 When platelet aggregation was initiated in the presence of NTG, dose-dependent inhibition was exhibited by NTG in both groups; however, the inhibition was significantly weaker in long-term smokers. Because the antiplatelet effects of NTG are mediated through its biotransformation to NO, previous studies reported long-term smoking.

The mechanism of potentiation of NTG-induced platelet inhibition in nonsmokers. NAS, which is identical to NAC except for the absence of the sulfhydryl group, did not potentiate NTG-induced platelet inhibition in saline rabbits, thus confirming that NAC-derived potentiation of NTG-induced inhibition is mediated by the interaction of NTG and the sulfhydryl group of NAC. In this study, NAC augmented the NTG-induced inhibition of platelet aggregation in nonsmokers. NAS, which is identical to NAC except for the absence of the sulfhydryl group, did not potentiate NTG-induced platelet inhibition in saline rabbits, thus confirming that NAC-derived potentiation of NTG-induced inhibition is mediated by the interaction of NTG and the sulfhydryl group of NAC. In the present study, NAC-derived potentiation was poor in both smokers and BSO-treated rabbits in which intraplatelet GSH was depleted. Our results suggest that decreased intraplatelet GSH may disturb not only intraplatelet biotransformation of NTG but also the formation of nitrosothiols (RSNO) through the interaction between NTG and thiols in plasma.33,35 In this study, NAC augmented the NTG-induced inhibition of platelet aggregation in nonsmokers. NAS, which is identical to NAC except for the absence of the sulfhydryl group, did not potentiate NTG-induced platelet inhibition in saline rabbits, thus confirming that NAC-derived potentiation of NTG-induced inhibition is mediated by the interaction of NTG and the sulfhydryl group of NAC. In the present study, NAC-derived potentiation was poor in both smokers and BSO-treated rabbits in which intraplatelet GSH was depleted. Our results suggest that decreased intraplatelet GSH may disturb not only intraplatelet biotransformation of NTG but also

<table>
<thead>
<tr>
<th>TABLE 3. ADP-Induced Increase in Intraplatelet cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers (n=10)</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>NTG</td>
</tr>
<tr>
<td>0 μg/mL</td>
</tr>
<tr>
<td>12.5 μg/mL</td>
</tr>
<tr>
<td>25 μg/mL</td>
</tr>
<tr>
<td>50 μg/mL</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD.

*P<0.01 and †P<0.001 compared with the absence of NTG.
‡P<0.05 and §P<0.01 compared with the absence of NAC.
|P<0.05 and ¶P<0.01 compared with nonsmokers.

Figure 2. Effects of NAC, NAS, and BSO on NTG-induced platelet inhibition in rabbits. Conditions were as follows: PRP obtained from the saline group (●), PRP obtained from the saline group with 1 mmol/L NAC (○), PRP obtained from the saline group with 1 mmol/L NAS (△), PRP obtained from the BSO group (□), PRP obtained from the BSO group with 1 mmol/L NAC (■), and PRP obtained from the BSO group with 1 mmol/L NAS (▲). *P<0.01 for saline vs BSO group. †P<0.05, ††P<0.01 for the absence vs presence of NAC.
intraplatelet metabolism of S-nitrosocysteine. In addition, NAC seems to not only impair platelet aggregation but also promote disaggregation, especially in the presence of a high concentration of NTG, even in smokers. Although further studies are needed to clarify the disaggregating effect of NAC, this might be independent of intraplatelet GSH.

In conclusion, our results indicate that NTG resistance occurs in long-term smokers, possibly due to a depletion of intraplatelet GSH. Thus, the adverse effects of long-term cigarette smoking are not only not limited to the pathophysiology of ischemic heart disease but are also involved in the development of resistance to therapeutic approaches with NTG.

References

In Table 4, we present data on the effects of NAC on the intraplatelet GSH content. NAC supplementation was found to significantly increase the intraplatelet GSH content in both smokers and saline rabbits compared to non-smokers and BSO rabbits.

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers (n=6)</th>
<th>Smokers (n=6)</th>
<th>Saline Rabbits (n=6)</th>
<th>BSO Rabbits (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total glutathione, mol/10^18 platelets</td>
<td>11.56±0.63</td>
<td>5.24±1.01*</td>
<td>10.78±1.87</td>
<td>4.76±2.15*</td>
</tr>
<tr>
<td>GSH, mol/10^18 platelets</td>
<td>11.51±0.62</td>
<td>5.19±1.01*</td>
<td>10.75±1.87</td>
<td>4.75±2.15*</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>241.4±37.2</td>
<td>124.1±15.5*</td>
<td>472.9±95.2</td>
<td>453.7±78.0</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD. *P<0.001 compared with nonsmokers or saline rabbits.

Note: *P<0.001 compared with nonsmokers or saline rabbits.

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