Thioredoxin as a novel sensitive marker of biological stress response in smoking

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Thioredoxin is a low molecular weight (approximately 12 kDa) redox protein, and protects against harmful stimuli such as oxidative stress. Smoking evokes oxidative stress, among other biological responses. The clinical relevance of thioredoxin in smoking has not been fully investigated. Here, we examined the effects of smoking on serum and urinary thioredoxin levels, in comparison with various stress markers. Serum thioredoxin levels in the smoking group (10 subjects) were significantly higher than those of the non-smoking group (5 subjects). After smoking, serum thioredoxin levels significantly decreased, while urinary levels significantly increased. On the other hand, the levels of serum and salivary cortisol, plasma norepinephrine, salivary amylase, salivary thioredoxin, and urinary 8-hydroxy-2'-deoxyguanosine levels before and after smoking were not significantly different. These results suggest that a decrease in thioredoxin in the serum and the concomitant increase in the urine is a novel sensitive marker of biological stress responses induced by smoking. The change seems to be evoked by mechanisms different from hormonal or 8-hydroxy-2'-deoxyguanosine-forming stress responses.

Key Words: smoking, thioredoxin, 8-OHdG, oxidative stress, stress marker

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moking is known to be a risk factor for cardiovascular diseases and cancer.1–5 Smoking evokes many acute and chronic biological changes in humans, and a variety of biomarkers such as cortisol, catecholamines, and amylase have been studied for monitoring the malicious effects of smoking. Several studies have shown that changes in the levels of serum and salivary cortisol are associated with smoking.6–7 However, because cortisol levels are influenced by circadian rhythms and various stresses,8–10 Smoking augments the levels of catecholamines including epinephrine.11–13 However, as catecholamine levels are largely influenced by exercise.14 The changes to salivary amylase in relation to smoking have been reported in several studies and the significance as a smoking marker is not determined.15–17

On the other hand, many studies have revealed that oxidative stress is associated with smoking. As a marker of oxidative damage, 8-epi-prostaglandin F2α (8-epi-PGF2α), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and F2-isoprostane (F2-IsoP) in plasma, serum or urine have been reported. 8-epi-PGF2α level is significantly higher among smokers than that in non-smokers.18–20 Several reports showed that urinary 8-OHdG is elevated in the smoking group,21–23 while one study reported no change.24 Most studies analyzed biomarkers for chronic changes caused by smoking but research on the acute changes is limited. Among the acute changes, F2-IsoP in plasma and urine was reported to increase significantly 1 h after smoking.25 However, the experimental procedure is complex, requiring sample pretreatment and gas chromatography-mass spectrometry.

Thioredoxin (TRX) is a low molecular weight (~12 kDa) redox protein, and acts as a protective protein against harmful stimuli such as oxidative stress.26–28 Intracellularly, TRX scavenges reactive oxygen species and regulates various signal transduction pathways in cellular activation and apoptosis.29 Extracellularly, TRX shows removal of reactive oxygen species, cytoprotective effects, and anti-inflammatory and anti-apoptotic functions.30–32 There are few studies on the control of TRX in plasma and urine. After the bolus injection of recombinant human TRX (rhTRX), the concentration of rhTRX in plasma decrease quickly, with the half-life of plasma rhTRX being 1.17 h. Further, when rhTRX (8 mg/kg/day = 0.33 mg/kg/h) was injected intravenously for 20 h in two animals, the excreted amounts of rhTRX were 483 and 1,110 μg (24% and 56% of the injected rhTRX) in urine, whereas the amounts in bile were 0 and 0 μg, respectively.33 These results suggest that TRX protein is degraded somewhere and is excreted in the urine.

A change in TRX level with smoking was reported in several studies. As for chronic changes, one report showed that TRX levels did not change after quitting smoking.34 In another report, TRX levels were significantly higher in the smoking group compared to those in the non-smoking group.35 The acute biological response of TRX in smoking has not been fully investigated.

In regard to cellular levels, TRX was reported to be secreted following stimuli such as oxidative stress through a leaderless export pathway.30 IL-1β is also secreted by leaderless pathways. Recently, a new mechanism of IL-1β release was identified.36 However, the precise molecular mechanism of the secretion of TRX has not been elucidated. Here, we examined the effects of smoking on serum and urinary TRX levels in order to investigate acute physiological responses in comparison with other stress markers. We demonstrated that the levels of serum and salivary cortisol, plasma norepinephrine, salivary amylase, salivary TRX, and urinary 8-OHdG levels did not change immediately after smoking. Urinary TRX increased, while serum TRX concomitantly decreased immediately after smoking. These results suggest that TRX is a novel oxidative stress marker of immediate smoking responses, not directly linked to 8-OHdG-associated oxidative stress.

Materials and Methods

Subject recruitment. This study was conducted with the approval of the Ethics Review Board of Tenri Health Care University, Japan (approval no. 107). The subjects were 15 healthy adult Japanese men (mean age 21.6 ± 1.45 years) who provided informed consent. The subjects were classified into a smoking group of 10 subjects who smoked daily, and a non-smoking group of 5 subjects with no smoking experience.

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All subjects were tested at 7 am while fasting and without drinking. The smoking group were requested to refrain from smoking after waking up. Samples of about 10 ml of morning urine, 15 ml of venous blood (EDTA tube, Venoject II blood collecting tube), and 2 ml of saliva were taken from the subjects after they rested for 5 min. After walking for 5 min to the outdoor smoking area, subjects of the smoking group smoked cigarettes (1 to 3). The tobacco brands were not standardized among smokers. Subjects of the non-smoking group walk to the adjacent outdoor area unaffected by sidestream smoke and waited for 5 min. Samples of about 10 ml of urine, 15 ml of venous blood, and 2 ml of saliva samples were taken again from the subjects after they returned to the laboratory on foot and rested for 5 min. All samples were centrifuged at 1,500 × g for 15 min, and the supernatants were stored at −80°C until use. Samples were directly used for each assay.

**General stress markers.** Serum and salivary cortisol concentrations were measured using the elecsys cortisol II kit and cobs 8000 e602 apparatus (Roche Diagnostics GmbH, Mannheim). Plasma catecholamine concentration was measured using HPLC-ECD-300 (Eicom Corporation, Kyoto, Japan). Salivary amylase concentration was measured using WAKO L-TYPE AMYLASE kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and LABOSPECT 008 apparatus (Hitachi High-Technologies Corporation, Tokyo, Japan).

**Oxidative stress markers.** Concentrations of serum, salivary and urinary TRX and urinary 8-OHdG were measured using the TRX ELISA kit (Redox Bio Science, Kyoto, Japan) and New 8-OHdG Check (Japan Institute for the Control of Aging, Shizouka, Japan), respectively, with the VersaMax ELISA Microplate Reader (Molecular Devices, CA). Urinary creatinine concentrations were measured using LABOSPECT 008 apparatus (Hitachi High-Technologies Corporation, Tokyo, Japan). Concentrations of urinary TRX and urinary 8-OHdG were corrected by urinary creatinine levels (ng/gCr).

**Statistical analyses.** The statistical analyses were performed using the Mann-Whitney U test or the paired t test. Stat flex ver. 6 (Artec Co., Ltd., Osaka, Japan) software was used for the statistical analyses, and p < 0.05 was considered significant.

**Results**

We compared general stress markers such as serum and salivary cortisol, plasma norepinephrine and epinephrine, and salivary amylase between the smoking and non-smoking groups. These parameters were not different between the groups (Table 1, Supplemental Fig. 1*). Next, we compared oxidative stress markers between the two groups. Urinary and salivary TRX, and urinary 8-OHdG levels were the same for both groups (Table 2, Supplemental Fig. 2*). The serum TRX levels in the smoking group were significantly higher than those in the non-smoking group (Table 2, Fig. 1).

Next, we examined the changes in general and oxidative stress markers immediately after smoking. There were significant differences in plasma epinephrine and serum and urinary TRX in the smoking group after smoking (Table 3 and 4, Fig. 2 and 3) (Supplemental Fig. 3 and 4*). Notably, the serum TRX levels decreased significantly after smoking, while the urinary TRX levels increased significantly (Table 4, Fig. 2 and 3).

We also monitored for changes in these markers in non-smokers.

### Table 1. Comparison of general stress markers between the smoking and non-smoking groups

<table>
<thead>
<tr>
<th></th>
<th>Serum cortisol (µg/dl)</th>
<th>Salivary cortisol (µg/dl)</th>
<th>Plasma norepinephrine (ng/ml)</th>
<th>Plasma epinephrine (ng/ml)</th>
<th>Salivary amylase (KU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Smoking</strong></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>14.07 ± 3.44</td>
<td>12.85 ± 2.43</td>
<td>0.42 ± 0.14</td>
<td>0.48 ± 0.25</td>
<td>3.9 ± 0.38</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Non-smoking</strong></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>12.85 ± 3.44</td>
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<td>0.42 ± 0.14</td>
<td>0.48 ± 0.25</td>
<td>3.9 ± 0.38</td>
</tr>
<tr>
<td>p value</td>
<td>0.2703</td>
<td></td>
<td>0.5403</td>
<td></td>
<td>0.0934</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. p value: smoking group (n = 10) vs non-smoking (n = 5); Mann-Whitney U test.

### Table 2. Comparison of oxidative stress markers between smoking and non-smoking groups

<table>
<thead>
<tr>
<th></th>
<th>Serum TRX (ng/ml)</th>
<th>Urinary TRX (ng/gCr)</th>
<th>Salivary TRX (ng/ml)</th>
<th>Urinary 8-OHdG (ng/gCr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Smoking</strong></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>31.6 ± 10.6</td>
<td>20.6 ± 3.9</td>
<td>1,027.7 ± 827.7</td>
<td>1,237.7 ± 1,177.3</td>
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<tr>
<td>p value</td>
<td>0.0071*</td>
<td>0.9025</td>
<td>0.5403</td>
<td>0.8065</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. p value: smoking group (n = 10) vs non-smoking (n = 5); Mann-Whitney U test. *p<0.01.

### Table 3. Smoking group: Comparison of general stress markers before and after smoking

<table>
<thead>
<tr>
<th></th>
<th>Serum cortisol (µg/dl)</th>
<th>Salivary cortisol (µg/dl)</th>
<th>Plasma norepinephrine (ng/ml)</th>
<th>Plasma epinephrine (ng/ml)</th>
<th>Salivary amylase (KU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before</strong></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>14.07 ± 3.44</td>
<td>13.38 ± 5.74</td>
<td>0.42 ± 0.14</td>
<td>0.38 ± 0.15</td>
<td>4.82 ± 0.14</td>
</tr>
<tr>
<td>p value</td>
<td>0.5071</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>After</strong></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>12.85 ± 3.44</td>
<td>12.85 ± 2.43</td>
<td>0.42 ± 0.14</td>
<td>0.48 ± 0.25</td>
<td>3.9 ± 0.38</td>
</tr>
<tr>
<td>p value</td>
<td>0.4981</td>
<td></td>
<td>0.4888</td>
<td></td>
<td>0.0199*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. p value: before smoking vs after smoking; paired t test. *p<0.05. n = 10.
We have demonstrated that serum TRX level was significantly higher in the smoking group compared to that in the non-smoking group. On the other hand, urinary 8-OHdG, a known oxidative stress marker,\(^{(21–23)}\) was not significantly changed between the smoking and the non-smoking groups. The current study indicates that serum TRX is a more sensitive biomarker of chronic oxidative responses associated with smoking than urinary 8-OHdG.

As previously reported,\(^{(11–13)}\) the level of plasma epinephrine increased. As the levels are also influenced by exercise,\(^{(14)}\) catecholamines are not specific markers for smoking. The cause of the change in plasma epinephrine was not clear. Walking to the smoking area might have caused the increase in plasma epinephrine.

A decrease in serum TRX and reciprocal urinary TRX increase was observed immediately after smoking. The reason for these changes remains unclear. As for the decrease of serum TRX, TRX protein may be modified by oxidative stress due to smoking, which leads to its subsequent degradation.\(^{(32)}\) Another possibility is the excretion of TRX in urine. There are few studies regarding the regulation of TRX in the kidneys. Kasuno \textit{et al.}\(^{(37)}\) reported that urinary TRX1 caused by oxidative stress is not derived from serum TRX1 filtered by glomeruli but from its excretion from tubular epithelial cells. Thus, it is assumed that TRX is derived from tubular epithelial cells in response to oxidative stress caused by smoking.

These results suggest that the decrease of serum TRX and the concomitant urinary increase is a novel sensitive marker of biological stress responses induced by smoking. These changes are putatively evoked by mechanisms other than hormonal or 8-OHdG-forming responses.

We could not standardize the tobacco brands or recruit individuals with similar smoking histories. Therefore, the tobacco brands used and the smoking history of the subjects might have affected the degree of change in these biomarkers levels.

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Author Contributions

Study concept and design: TS, HM, EI-K and TI. Acquisition of data: TS, HM and CH. Analysis and interpretation of data: TS, HM, CH, EI-K and TI. Drafting of the manuscript: TS and HM. Statistical analysis: TS. Technical: TS, HM and CH. Study supervision: HM, EI-K and TI.

Acknowledgments

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Abbreviations

8-epi-PGF2α  8-epi-prostaglandin F2α
F2-IsoP  F2-isoprostane
8-OHdG  8-hydroxy-2-deoxyguanosine
TRX  thioredoxin

Conflict of Interest

No potential conflicts of interest were disclosed.

References