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Biochemical and Biophysical Research Communications 293 (2002) 517–520

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# Psychological stress increases bilirubin metabolites in human urine☆

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Received 25 March 2002

**Abstract**

Some authors have suggested that psychological stress induces the production of reactive oxygen species (ROS). Some studies have supported that bilirubin exerts anti-oxidative effects in vivo. However, it is not known whether ROS induced by psychological stress provoke bilirubin oxidation in vivo. We investigated if the concentration of bilirubin oxidative metabolite (BOM), a bilirubin oxidative metabolite, increased in urine from subjects exposed to psychological stress. Sixty healthy male volunteers working in a pharmaceutical company were divided into a Group I which did not attend a conference, a Group II which attended a conference but did not deliver a speech, and a Group III which attended a conference and delivered speeches in the presence of the company executives. Subjective stress was scored (self-rating score) after subjects in Group III delivered their speeches at the conference. Urine was collected on the next day. The BOM concentrations, as measured by enzyme-linked immunosorbent assay, were normalized to the urinary concentration of creatinine. The concentration of BOM in Group III was significantly higher compared to that in Groups I and II ($p < 0.01$ and $p < 0.05$, respectively). Furthermore, in Group III, the concentration of BOM correlated with the self-rating stress score ($r = 0.53$, $p < 0.01$). These findings suggest that emotional stimuli are associated with an increase in the oxidative metabolites of bilirubin in human urine, and that BOMs could be useful markers of psychological stress. 

*Keywords:* Bilirubin; Psychological stress; 24G7 anti-bilirubin monoclonal antibody; Bilirubin oxidative metabolite; Reactive oxygen species; Stress marker

Some studies have suggested that psychological stress induces the production of reactive oxygen species (ROS) [1,2]. For example, Adachi et al. [1] have reported that the oxidized product of the bases in nuclear DNA in rat livers, that is, 8-hydroxy-2′-deoxyguanosine (8-OH-dG) increases, after exposure to psychological stress, like conditioned emotional stimuli in a communication box. To our knowledge, this is the first evidence that psychological stress might increase the production of ROS in vivo, which introduces 8-OH-dG into the DNA bases. Furthermore, Irie et al. [2] suggested that psychological stress, as in the case of conditioned taste aversion experiments [3], significantly increased the formation of 8-OH-dG, a known oxidative DNA modification relevant to renal cell carcinoma. In other studies, chronic and sub-chronic emotional stress was shown to enhance oxidative stress caused by an increase in plasma superoxide, and to modify the mechanisms of anti-oxidative effects in humans [4]. Moreover, immobilization-induced stress causes oxidative damage to lipids, proteins, and DNA in the brain of rats, although this type of stress may be a combination of psychological stress and psycho-physical stress [5]. After an emotional stimulus, the

☆ *Abbreviations:* 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; ROS, reactive oxygen species; BOM, bilirubin oxidative metabolite; ELISA, enzyme-linked immunosorbent assay; CMI, Cornell Medical Index; SDS, Self-rating Depression Scale; HO, hemeoxygenase; IQR, interquartile range.

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0006-291X/02/$ - see front matter 
PII: S0006-291X(02)00233-4

level of catecholamines increases in the cerebral circulation [6] and it induces sudden increases in systolic blood pressure which could lead to the rupture of cerebral vessels with hyalinotic or atherosclerotic alterations [7]. Other relevant studies have indicated that psychological stress influences ROS production and subsequent oxidative stress contributing to the neuronal degeneration and cell death that occur during cerebral ischemia-reperfusion injury [8–11]. Bilirubin is biosynthesized from heme, catalyzed by heme oxygenase (HO) [12] and biliverdin reductase [13,14], and it is thought to be a harmful and useless substance in the body. High concentrations of unconjugated bilirubin in the neonatal plasma are associated with the risk of bilirubin encephalopathy and neuronal injury [15]. However, in contrast to its toxic effects, Stocker et al. [16] have reported that bilirubin can act as a powerful antioxidant in vitro. It has also been reported that bilirubin has strong, protective functions in vivo when the mechanisms of defence against oxidative stress are challenged [17,18]. The reaction of bilirubin with ROS should result in the production of many kinds of bilirubin oxidative metabolites (BOMs). We have detected several types of BOMs in human urine using an anti-bilirubin monoclonal antibody designated 24G7 [19,20]. We have previously determined the chemical structures of 2 BOMs (biotripyrrin-a and -b) purified from the urine of healthy volunteers [21].

Several lines of evidence support the role of bilirubin in the prevention of oxidative damage-associated cardiovascular disease [22–28]. For example, immunohistochemical examination of HO-1 and bilirubin in atherosclerotic regions in hypercholesterolemic rabbits, using the anti-bilirubin antibody 24G7, demonstrated accumulation of bilirubin and/or BOMs in foam cells, indicating that heme was actually degraded into bilirubin, which suppressed the progression of atherosclerosis and endothelial dysfunction in atherosclerotic lesions [25]. The urinary concentrations of BOMs increased in patients who had undergone laparotomy [18,29,30], and also in rats under oxidative stress, such as treatment with endotoxin [17,31] or liver ischemia-reperfusion [32]. Furthermore, we have also indicated that the concentration of bilirubin and BOMs increased in the cerebrospinal fluid of patients with Alzheimer's disease [33], supporting the hypothesis that ROS might be involved in the onset of the disease [34]. These reports suggest that ROS produced under different oxidative conditions are broken down by bilirubin.

Based on these reports on the possible relationship among psychological stress, oxidative stress, and bilirubin as the antioxidant, we examined the possibility that psychological stress contributed to the oxidative conditions, and the subsequent increase of the urinary concentration of BOMs produced by the reaction of bilirubin with ROS.

## Methods

### *Subjects and experimental design*

*Speech stress*. Sixty healthy male volunteers from a pharmaceutical company, 36–50 years old (mean $\pm$ SD $= 41.5 \pm 3.1$ years) participated in the experiment. They were classified into a Group III consisting of subjects ($N = 32$) who had to deliver a speech, of about 30 min, on how they would further develop their company. The company executives evaluated both the gist of the speech and the answers to their questions after each speech; a Group II of subjects ($N = 19$) who attended the conference but did not deliver a speech, and a Group I ($N = 9$) of subjects who did not attend the conference. There was no significant difference in age, grade, or number of subjects who smoked among these groups. Stress was scored on the day of the conference (self-rating score) and urine was collected on the following day. It is especially important to investigate the correlation between the self-rating stress scores of the subjects subjected to stress and their BOM levels.

*Subjective parameters of psychological stress*. The subjects were asked to score any situation of stress they had. Scores of 1+, 2+, 3+, and 4+ indicated no stress, slight stress, moderate stress, and severe stress, respectively.

### *Measurements*

Urine samples were stored at −80 °C until assayed. Urinary BOMs were measured in duplicate using ELSIA that employs 24G7 anti-bilirubin monoclonal antibody, and the results were corrected for the urinary concentration of creatinine, which was determined with Accuras Auto CRE (Shino-test, Tokyo). The 1.0 relative value of the BOM level denotes the mean of the control group (Group I) in Fig. 1.

### *Psychological tests*

The subjects were evaluated using the Cornell Medical Index (CMI) and Self-rating Depression Scale (SDS), and the results showed that they were all within normal ranges for psychoneurosis and depression.

### *Statistical analyses*

Differences between two groups were tested using Mann–Whitney test and those among the three groups by both Kruskal–Wallis test and Mann–Whitney test. Correlations were tested using Spearman's correlation coefficient. Logarithmic transformation was applied and checked again for normality when SD was calculated. A $p$ value of $<0.05$ was considered as statistically significant.

## Results and discussion

Since in the preliminary experiment we observed that the concentration of BOM increased on the day after a speech was delivered at a conference, we subjected the volunteers ($N = 32$, Group III) to the same stimulus, that is, a speech on a special theme and determined the concentration of BOMs in urine on the following day to confirm the effects of such stressful situation.

The concentration of BOMs in Group III (relative value $= 1.7$) was significantly higher than that in Group II (relative value $= 1.5$; $p < 0.05$) and Group I (relative value $= 1.0$; $p < 0.01$). The difference between Groups I and II was also significant ($p < 0.05$) (Fig. 1). A signif-

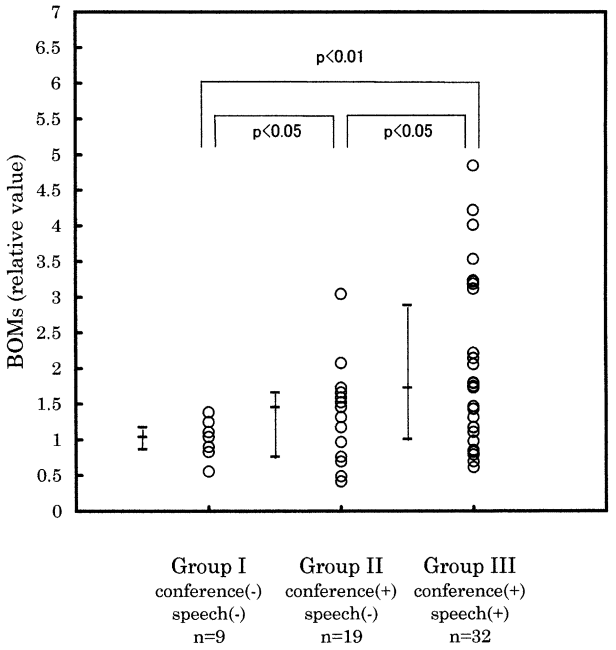


Fig. 1. Urinary BOMs of Groups I, II, and III. Group III attended the conference and gave a speech; Group II attended the conference but did not give a speech; Group I did not attend the conference. 1.0 denotes the mean of Group I, and the vertical bars represent the medians and IQRs.

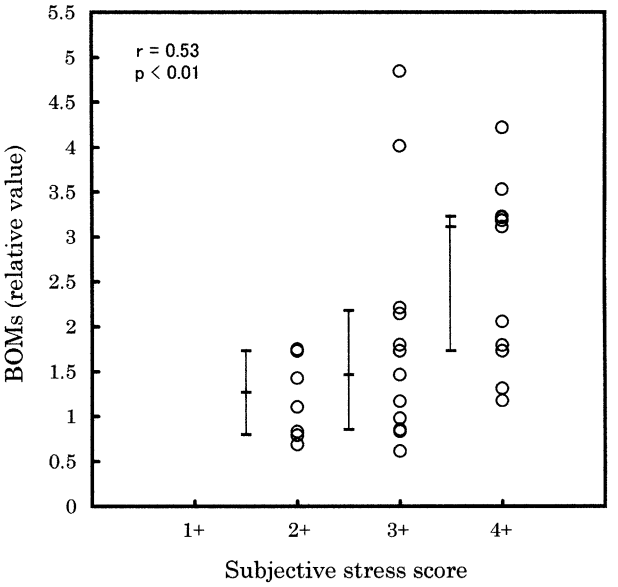


Fig. 2. Correlation of the concentration of urinary BOMs with self-rating stress scores in Group III. 1.0 denotes the mean of Group I in Fig. 1, and the vertical bars represent the medians and IQRs.

icant correlation was observed between stress scores and the concentration of urinary BOMs in Group III ($r = 0.53$, $p < 0.01$) (Fig. 2).

It is of interest to note that the median and interquartile range (IQR) of the relative value of BOMs in Group II were larger than those in Group I and that there was a significant difference between Groups I and II ($p < 0.05$). We speculated that the subjects were influenced by the surroundings at the conference even though the subjects of Group II did not participate as speakers.

The site in which 8-OH-dG is produced by the reaction with DNA and ROS is mainly defined as the nucleus of the cells [1]. Bilirubin exists in blood bound to the hydrophobic sites of both albumin and lipoproteins [35], and it is also distributed in the membranes of mitochondria and microsomes in the cells (unpublished data). Therefore, bilirubin itself is distributed all over the body and is degraded to BOMs when reacting with ROS, which arise not only in the systemic circulation but also at the cellular level in each organ of the body; thereafter BOMs are immediately excreted into the urine. In the present study, we showed that acute psychological stress was associated with an increase of urinary BOMs in humans. In a recent report, Cernak et al. [4] demonstrated that chronic psychological stress (political intolerance, awareness of potential military attack) led to an increase of plasma superoxide and malondialdehyde as final products of lipid peroxides.

With respect to psychological stress, the metabolic process of ROS production leading from the homeostatic stage to the oxidative stage in the body remains unknown. Especially, it is difficult to decide whether the increase of urinary BOMs associated with psychological stress reflects an increased protection of ROS in the whole body or in only parts of it. For example, it has been reported that emotional stimuli is derived from the cerebral cortex and/or the limbic system. Therefore there is a possibility that acute psychological stress might induce mild cerebrum ischemia-reperfusion injuries and oxidative stress, which would subsequently result in the production of ROS in the cerebrum. These ROS would react with bilirubin leading to an increase of BOMs [7–11,35]. Although the identification of each BOM increased in urine from subjects exposed to psychological stress (speech stress) is now in progress, the concentrations of BOMs except for biotripyrrin-a and -b [17,21] seemed to be elevated (unpublished data).

In summary, we have shown that urinary BOMs, i.e., the oxidative products of bilirubin, increased immediately after the volunteers were subjected to a situation of psychological stress, and that the concentration of the urinary BOMs correlated with self-rated stress scores. These findings suggest that BOMs may serve as markers of psychological stress. Furthermore, the physical and mental burdens associated with the test are mild, because the method is not invasive.

## Acknowledgments

We would like to thank Masataka Ara, Shinya Sasaki, and Hajime Yoshimura for their excellent technical assistance.

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