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Letter

Effects of rofecoxib on lipid oxidation in plasma and aortas of rats

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ABSTRACT — A selective cylooxygenase-2 (COX-2) inhibitor, rofecoxib, was withdrawn from the worldwide market due to an increased risk of cardiovascular (CV) events. A hypothesis has been proposed that rofecoxib promotes lipid oxidation, which increases the risk of CV events. However, this hypothesis was only predicated on *in vitro* experiments using isolated human low density lipoprotein and diluted human plasma. In the present study we investigated the effect of rofecoxib on the *in vitro* and *in vivo* production of thiorbarbituric acid reacting substance (TBARS) as an indicator of oxidation in plasma and aortas in rats. *In vitro* experiment, the TBARS production in plasma and aortic homogenate was not changed by the addition of rofecoxib at 2 μM, which concentration is around the maximum plasma concentration at clinical doses, or even at 200 μM. In addition, the production was not increased by rofecoxib in the presence of $FeSO_4$ as a typical oxidant. Meanwhile the TBARS production in the aorta of rats after 4-weeks administration of 10 mg/kg/day rofecoxib was comparable to that of the control rats. These results *in-vitro* and *in-vivo* experiments suggest that rofecoxib would have no or very weak effect on lipid oxidation in clinical usage, and it is thought that the increase of CV events already reported stemmed from causes other than oxidative stress.

Key words: Rofecoxib, Oxidation, Lipids, Selective cyclooxygenase-2 inhibitors, Cardiovascular events

INTRODUCTION

A highly selective cyclooxygenase-2 (COX-2) inhibitor, rofecoxib was marketed as VIOXX® by Merck & Co. beginning in 1999 and was used widely in many countries for the treatment of acute pain and osteoarthritis. However, in 2004, rofecoxib was withdrawn from the worldwide market due to an increased risk of cardiovascular (CV) events such as heart attack and stroke among patients who had received long term rofecoxib treatment (Bombardier *et al.*, 2000; Ray *et al.*, 2002; Bresalier *et al.*, 2005; Baron *et al.*, 2008). Several hypotheses for the increased CV risk by rofecoxib have been proposed. McAdam *et al*. (1999) proposed that the balance between thromboxane A_2 (a vasoconstrictor and promoter of platelet aggregation) in the platelets, and prostaglandin I_2 (a vasodilator and inhibitor of platelet aggregation) in the vascular endothelium was disturbed by selective inhibition of COX-2 by rofecoxib, leading to the CV events. Mason *et al*. (2006, 2007) and Walter *et al*. (2004) demonstrated that a pro-oxidant effect of rofecoxib promote oxidative damage to low density lipoprotein (LDLs) isolated from human plasma, and proposed the oxidation hypothesis. Liu *et al*. (2010) reported that rofecoxib treatment for 2 months induced more than 100-fold increase of plasma concentration of 20-hydroxyeicosatetraenoic acid (20-HETE), one of the arachidonic acid metabolites, which shortened of bleeding time, in mice. They also pointed out the possibility that these changes were associated with the increase of CV risk. Oitate *et al.* (2007) demonstrated the disruption of elastic lamellae in the aortic wall of rats treated with rofecoxib for 4 weeks. We also demonstrated that rofecoxib deteriorates vasoregulation with the disruption of elastic lamellae in aortic wall (Miyajima *et al.*, 2013). Regardless of those previous researches, the mechanism of the increased risk of CV events has not been clarified entirely.

In the oxidation hypothesis proposed by Mason *et al*. (2006, 2007) and Walter *et al*. (2004) it was suggested that rofecoxib would induce and/or promote lipid oxida-

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tion. Meanwhile Reddy and Corey (2005) demonstrated that rofecoxib was ionized under physiological conditions to an anion, followed by reacting readily with free oxygen to form a reactive maleic anhydride derivative, which might oxidize proteins. Or it was thought possible that rofecoxib could promote oxidation, like ascorbic acid does in the Fenton reaction with iron and copper ions (Nelli *et al*., 2009; Gutteridge, 1995). However, the oxidative effect of rofecoxib has not been verified yet in the biomaterial by a third party.

In this study, to clarify whether rofecoxib has oxidative potentials *in vitro* and on *in vivo*, we investigated the effect of rofecoxib on the production of thiorbarbituric acid reacting substance (TBARS) in plasma and aortic homogenate from rats, and further examined the TBARS production in the aorta after 4-weeks administration of rofecoxib to rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from Japan SLC Inc. (Shizuoka, Japan) and used after 7 or more days of acclimatization. All rats were housed in a temperature (23 ± 1°C) and humidity (55 ± 5%)-controlled room with 12-hr light/dark cycle. Water and diet were available ad libitum throughout the study. All experimental animals were handled in accordance with the institutional and national guidelines for the care and use of laboratory animals.

In vitro oxidation assay

Blood samples were collected from the inferior vena cava vein of each rat (10 weeks old) with a heparinized syringe under isoflurane anesthesia. Plasma samples were subsequently obtained by centrifugation at 3,000 *g* at 4°C for 10 min. After blood collection, the thoracic aorta was isolated after euthanasia by exsanguination under isoflurane anesthesia. The aorta was prepared with 20 times its volume of 10 mM phosphate buffered saline (pH 7.4) (PBS). Both the plasma and homogenate (5%) were used for the *in vitro* assay.

Rofecoxib, celecoxib, etoricoxib and valdecoxib were kindly given by Daiichi Sankyo Company Ltd. (Tokyo, Japan), and each was dissolved in dimethylsulfoxide for experimental use. To evaluate the oxidative effect of rofecoxib and the other COX-2 inhibitors, the following *in vitro* assay was conducted. For evaluation of direct oxidative activity, plasma (final concentration 80%) and aortic homogenate (final concentration 4%) were incubated with rofecoxib (2 or 200 μM) or $FeSO_4$ (400 and 1200 μM for plasma and 100 and 400 μM for aortic homogenate) as a positive control, at 37°C for 1.0 hr (n = 3). For evaluation of pro-oxidant activity, the same treatment was performed in the presence of $FeSO_4$ (400 μM for plasma and 100 μM for aortic homogenate), to which (+)-ascorbic acid (10 mM for plasma and 500 μM for aortic homogenate) was added as a positive control for the Fenton reaction instead of the COX-2 inhibitors. Celecoxib (4 μM; Celecox® product information, Astellas Pharma Inc., Apr 2014), valdecoxib (1 μM: BEXTRA®, product information, Pfizer, July 2003) and etoricoxib (8 μM, Rodrigues *et al*., 2003) were also used for the experiments. The concentration of each COX-2 inhibitor was about the same as the maximum concentration when used clinically or in clinical trials, except for 200 μM rofecocxib. The final dimethylsulfoxide concentration was 1.0% in all reaction mixtures. To 70 μL of the reaction mixture 10 μL each of ethylenediaminetetraacetic acid･Na in pure water (18 mM) and dibutyl hydroxy toluene (180 μM) in methanol were added to stop the reaction. These samples were used for the thiobarbituric acid reactive substances (TBARS) assay, which was performed using the TBARS assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) as described previously (Miyajima *et al*., 2015). The TBARS production was evaluated as the malondialdehyde (MDA) concentration.

In vivo administration study

Eight rats (5 weeks old) were divided into two groups, rofecoxib treated and control. In the rofecoxib treated group, 10 mg/kg/day rofecoxib was administered orally for 4 weeks, while in the control group vehicle (polyethylenglycol 400) was administered. At 24 hr after the last treatment, the thoracic aorta was isolated after euthanasia by exsanguination under isoflurane anesthesia. The aortic homogenate (0.3%) was prepared as described above and used for the TBARS assay. The assay was performed as described above, except that the MDA-TBA adduct was detected fluorometrically at an excitation wavelength of 530 nm and an emmision wavelength of 550 nm using a spectrophotometer F-2500 (Hitachi High-Technologies Corporation, Tokyo, Japan).

Statistical analyses

Student's t-test and Dunnett's multiple comparison test were used for statistical analysis (SPSS 17.0, IBM, Armonk, NY, USA). Differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

The effect of rofecoxib on the production of TBARS as an indicator of oxidation was evaluated in aorta and plasma from rats. The TBARS production in aortic homogenate was not affected by the addition of rofecoxib at 2 μM, which concentration was around the maximum plasma concentration in clinical usage (Schwartz *et al*., 2000), or even at 200 μM ($p < 0.05$, Fig. 1A). Meanwhile the production was significantly enhanced by the addition of $FeSO_4$, which is a typical initiator of oxidation. These results suggested that rofecoxib had very weak or no oxidation potential. In addition, to evaluate the pro-oxidant activity TBARS production in the presence or absence of rofecoxib was measured in aortic homogenates in which the production was stimulated by the addition of $FeSO_4$. Neither 2 μM nor 200 μM rofecoxib boosted the TBARS production (Fig. 1B). These results suggested that the pro-oxidant effect of rofecoxib was very weak or not. The experiments with plasma gave similar results to those in aortic homogenates (Fig. 2). Moreover, to verify oxidative potential *in vivo* the TBARS production was evalu-

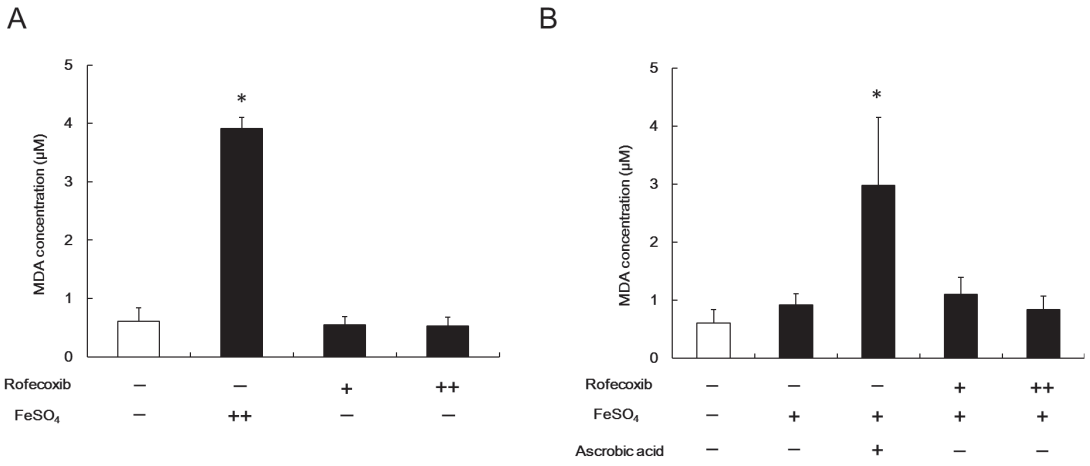

Fig. 1. Effect of rofecoxib on TBARS production in rat aortic homogenate *in vitro*. The oxidative activity (A) and pro-oxidative activity (B) were evaluated using aortic homogenate exposed to rofecoxib (-: 0, +: 2 or ++: 200 μM), $FeSO_4$ (-: 0, +: 100 or ++:400 μM) and/or ascorbic acid (-:0, +: 500 μM). Data are expressed as mean MDA concentration ± S.D., $n = 3$. *$p < 0.05$ vs. control (open bar in each graph) analyzed by Dunnett's multiple comparison test.

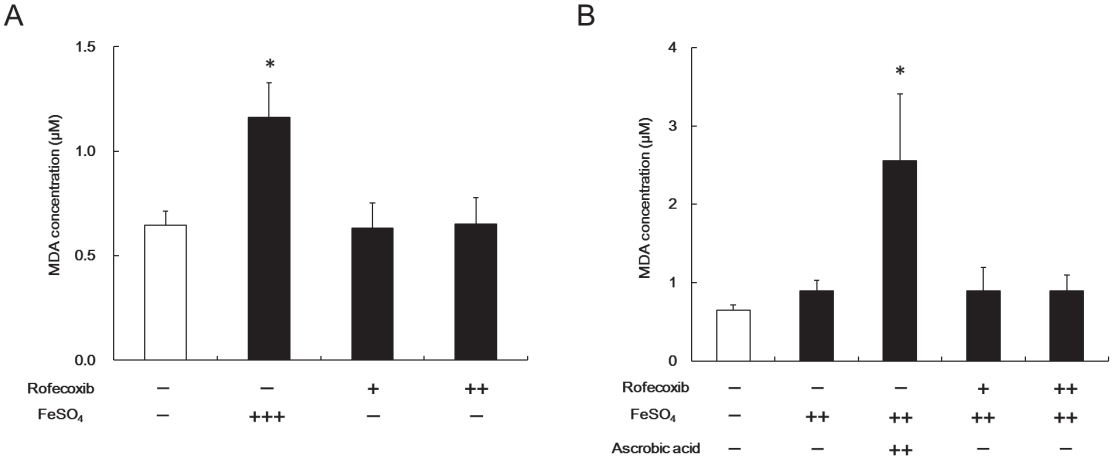

Fig. 2. Effect of rofecoxib on TBARS in rat plasma *in vitro*. The oxidative activity (A) and pro-oxidative activity (B) were evaluated using plasma exposed to rofecoxib (-: 0, +: 2 or ++: 200 μM), $FeSO_4$ (-: 0, ++: 400 or +++:1200 μM) and/or ascorbic acid (-: 0, ++: 10 mM). Data are expressed as mean MDA concentration ± S.D., $n = 3$. *$p < 0.05$ vs. control (open bar in each graph) analyzed by Dunnett's multiple comparison test.

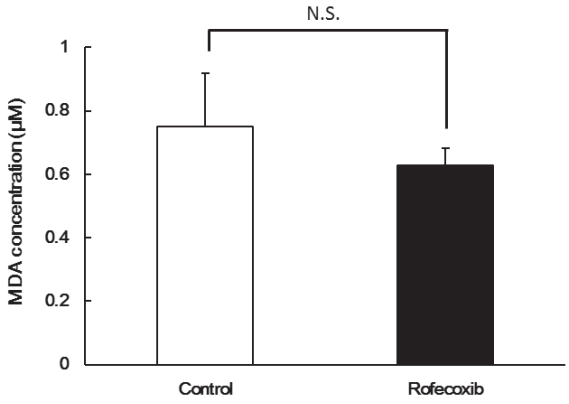

Fig. 3. Effect of repeated administration of rofecoxib on TBARS in rat aortas. Ten mg/kg/day Rofecoxib was administered orally to rats for 4 weeks and then thoracic aorta was isolated. TBARS production in the aortic homogenate was measured. Data are expressed as mean MDA concentration ± S.D., $n = 4$. N.S.: not significant (Student's *t*-test).

ated in aorta of rats after 4-weeks repeated administration of rofecoxib. The TBARS production in the aorta was comparable in rofecoxib treated and control groups (Fig. 3). Also in plasma the TBARS production was not changed after the same treatment in the preliminary study. We previously reported that the 4-week repeated administration of rofecoxib caused the dysfunction of the vasoregulation with elastin degradation in rat aortas. So such impairment in aorta was found not to be due to the oxidation. Those *in-vitro* and *in-vivo* results suggested that the oxidative potential of rofecoxib was too weak to cause the oxidative damage to the aortas, if any.

Our present results from *in vitro* experiments are inconsistent with the reports by Mason *et al*. (2006, 2007) and Walter *et al*. (2004) which demonstrated the oxidative effects by rofecoxib on human LDLs. As one of the reasons for the discrepancy, anti-oxidative substances such as vitamin E and glutathione would have been present in the plasma and aortic homogenates freshly prepared from rats in the present study, and could have cancelled out any oxidative effect by rofecoxib. Walter *et al*. (2004) demonstrated that an oxidative effect of rofecoxib occurred by *in vitro* experiments measuring TBARS production from isolated human LDL and oxygen radical absorption capacity assay with plasma diluted 800-fold with phosphate buffered saline. These experimental materials might have little or no antioxidants. Considering that living material under aerobic conditions have anti-oxidative ability in general, it is suggested that any oxidative effect of rofecoxib would be too weak to overwhelm that ability *in vivo*, even if it has some oxidizing ability.

After the withdrawal of rofecoxib from the market, it was reported that other selective COX-2 inhibitors (*e.g.* etoricoxib, parecoxib and valdecoxib) may also have the potential to increase CV risk (Aldington *et al*., 2005; Nussmeier *et al*., 2005). In the present study, however, neither celecoxib, etoricoxib nor valdecoxib affected TBARS production in rat aortic homogenate either in the presence or absence of $FeSO_4$ (data not shown). It was also suggested that these COX-2 inhibitors have no or very weak oxidative potential, too.

In conclusion, rofecoxib and the other COX-2 inhibitors used in the present study are suggested to have no or very weak oxidative potential on lipid oxidation in plasma and the aorta.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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