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Estimation of hydroxyl radical generation by salicylate hydroxylation method in multiple organs of mice exposed to whole-body X-ray irradiation

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Abstract
Appropriate experimental conditions for the estimation of hydroxyl radical generation by salicylate hydroxylation were determined for multiple organs of X-irradiated mice *in vivo*. The *in vitro* experiments showed that there were significant correlations between the salicylic acid (SA) concentration, the amount of 2,3-dihydroxy benzoic acid (2,3-DHBA) and the X-ray exposure dose, and we obtained two linear-regression equations to calculate the amounts of hydroxyl radicals generated by the X-irradiation. The optimum dosage of SA and the appropriate sampling time for *in vivo* experiments was determined, and significant increases in the ratio of 2,3-DHBA to SA were detected in several organs of mice after X-irradiation. The hydroxyl radical equivalents of the 2,3-DHBA increases were also calculated. Our results clearly demonstrated the usefulness of the salicylate hydroxylation method in estimating hydroxyl radical generation in multiple organs *in vivo*.

Keywords: *Hydroxyl radical, salicylic acid, mice, whole-body irradiation, X-ray*

Abbreviations: *SA, salicylic acid; ASA, acetylsalicylic acid; 2,3-DHBA, 2,3-dihydroxy benzoic acid; 2,5-DHBA, 2,5-dihydroxy benzoic acid*

Introduction

The measurement of reactive free radicals, such as hydroxyl radical, is generally difficult because of their high reactivity and extremely short lifetimes. In particular, it is almost impossible to detect or measure hydroxyl radical directly in *in vivo* experimental systems, therefore determinations of hydroxyl radical using several trapping agents are widely accepted. The two types of methods have been reported for detecting hydroxyl radicals *in vivo*. The first are relatively direct methods in which the extracts of spin adducts formed with spin trapping agents were analyzed by electron spin resonance (ESR). For example, Ghio et al. used ESR coupled with the spin trap α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone to demonstrate *in vivo* radical production in the lung of rats after asbestos exposure [1]. The second are the indirect methods that use some types of indicator of hydroxyl radical generation

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such as aromatic hydroxylation, and salicylate has become a popular trapping agent for hydroxyl radicals *in vivo*. Schapira et al. reported that hydroxyl radical generation could be detected by using aromatic hydroxylation in a similar asbestos exposure experiment *in vivo* [2].

Salicylic acid (SA) was first used as a trapping agent for detecting hydroxyl radical in *in vivo* experimental systems in 1986 by Floyd et al. [3], who reported hydroxyl radical generation in the several types of tissues of rats that had been treated with adriamycin. In the same year Grootveld and Halliwell also reported that hydroxyl radical generation could be detected in human body fluids by using salicylate hydroxylation [4]. This method has been successfully applied to demonstrate the relation between hydroxyl radical generation and pathology in many different organs, such as heart [5], brain [6], lung [7], intestinal tract [8], kidney [9], central nervous system [10] and was also employed to evaluate the possible involvement of hydroxyl radicals in diabetes [11] as well as in a sepsis model [12].

SA distributed in organs is metabolized by conjugation to form salicyl acyl glucuronide, salicyl phenolic glucuronide and salicyluric acid, and by enzymatic hydroxylation through cytochrome P450s to form 2,5-dihydroxy benzoic acid (2,5-DHBA). About 60% of salicylate remains unmodified and can undergo hydroxyl radical attack to produce 2,3-dihydroxyl benzoic acid (2,3-DHBA), a compound that has not been reported as a product of enzymatic metabolism. Thus 2,3-DHBA is thought to be a useful indicator of *in vivo* hydroxyl radical generation [13].

On the other hand, several studies have reported problems with this method. Montgomery et al. [14] encountered a problem when using SA to trap hydroxyl radicals because of unexpected amounts of metabolites of SA from non-biological sources, and they pointed out the need to consider artifactual production by components of the experimental apparatus. Kaur et al. [15] reported that in addition to hydroxyl radical, salicylate hydroxylation was also induced by peroxynitrite. Blackburn et al. found that the increase in 2,3-DHBA could not be detected in an enteritis mice model induced by dextran sulfate sodium administration because of changes in the metabolic capacity for SA of mice organs caused by the inflammatory responses themselves [16]. Moreover, almost all of the research involving *in vivo* experiments adopted similar experimental conditions in which the analyzes were made at 1 h after the administration of 100 mg/kg of SA [2,4,5], however, little data are available concerning the distribution and metabolism of SA *in vivo*. In addition, there are no reports on the quantitative relationship between the amounts of hydroxyl radicals and hydroxylated metabolites of SA even in *in vitro* experimental systems.

In the present study, the quantitative relationships among the concentration of SA, the amount of increase in 2,3-DHBA and the exposure dose of X-ray irradiation were determined in *in vitro* experiments. In addition, the levels of SA and 2,3-DHBA in multiple organs of mice were examined in order to determine the optimum dosage of acetylsalicylic acid (ASA) and the appropriate sampling time for *in vivo* experiments. Moreover, the increases in hydroxyl radicals corresponding to the increases in 2,3-DHBA were estimated in the multiple organs of mice that received whole body X-ray irradiation, using the relation determined in our *in vitro* experiments. Our results demonstrated the usefulness of the salicylate hydroxylation method for determination of hydroxyl radical levels in *in vivo* experimental systems.

Materials and methods

Chemicals

SA and ASA were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). 2,3-DHBA was obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). All chemicals used were the highest quality commercially available.

Animals

ICR male mice 8 weeks of age were purchased from SLC Inc. (Japan). The mice were fed a standard cubed diet (MF, Oriental Yeast Co., Japan) maintained on distilled water *ad libitum* and exposed to a 12 h light (7:00 AM to 7:00 PM) and 12 h dark (7:00 PM to 7:00 AM) cycle. Ambient temperature during the study was maintained at about 21°C. All procedures employed in the present study followed the guidelines for animal treatment of Kitasato University, which meet the "Principles of laboratory animal care", based on the Scientist's Center for Animal Welfare Consensus Recommendations on Effective Institutional Animal Care and Use Committees (Laboratory Animal Science. Special Issue. pp. 11–13, January, 1987).

Determination of SA and 2,3-DHBA

SA and 2,3-DHBA were measured according to the methods of Tsai et al. [17] and Yamamoto et al. [18] with some modifications. Twenty microliters of HCl (1 M) were added to 100 μl of serum placed in a 10 ml glass tube. The resulting solution was extracted with 3 ml of diethyl ether by mixing thoroughly for 1 min and was then centrifuged. The organic phase was collected, evaporated to dryness under nitrogen, and dissolved with 300 μl of mobile phase (30 mM sodium citrate/27.7 mM sodium acetate, pH 4.75). For determination of SA and 2,3-DHBA in the organs, tissue (within 0.5 g) from each mouse was homogenized with

ice-cold saline (0.5 ml), and deproteinized by addition of 10% (v/v) perchloric acid containing 1 mM EDTA–2Na and 100 μM sodium pyrosulfite (1.5 ml). HCl (1 M, 0.4 ml) was added to the supernatant fraction obtained by centrifugation. The resulting solution was extracted with 10 ml of diethyl ether, and analyzed as described above.

According to the method of Yamamoto et al. [18], the samples were analyzed using a Gilson high performance liquid chromatography (HPLC) system equipped with a Model 307 pump, and sigma 875 electrochemical detector (ECD; IRICA, Japan). Reverse-phase HPLC analysis was carried out using a YMC-Pack ODS-A A-312 column (150 × 6.0 mm, YMC Co., Ltd, Kyoto, Japan). The glassy carbon working electrode was set at a potential of +0.85 V, and the flow rate was 1.0 ml/min.

X-ray irradiation of the SA solution or experimental animals

The mice were exposed to X-ray irradiation (24 Gy, 2.22 Gy/min) using an X-ray generator (MS-30-5, Hitachi, Japan) equipped with 0.5 mm Cu and 1.0 mm Al filters. The irradiated mice did not show any clinical symptoms during the term of the experiments. The SA solution in a glass tube was also exposed to X-rays under the same conditions.

In vitro experiments

A solution of SA dissolved in 100 mM Tris–HCl buffer (pH 7.2) at a concentration of 100 μg/ml was exposed to 3–24 Gy of X-rays, and then measured the concentrations of SA and 2,3-DHBA by HPLC–ECD analysis. Furthermore, solutions of SA at 10–500μg/ml in the same buffer were exposed to 24 Gy of X-ray irradiation and analyzed in the same manner as above.

In the ESR study, the same buffer including 200 mM 5,5-dimethyl-1-pyrroline-1-oxide (DMPO; Dojin, Japan) was exposed to 3–24 Gy of X-rays. At 1 min after the irradiation, X-band ESR spectra in the first-derivative presentation were recorded using a JES-FA 100 spectrometer (JEOL Ltd, Tokyo, Japan). ESR measurements were performed under the following conditions: modulation frequency, 9.4 GHz; field modulation, 100 kHz; modulation amplitude, 0.1 mT; microwave power, 4 mW; center field, 335.6 mT; sweep width, 5 mT; sweep time, 2 min; and time constant, 0.03 s. All spectra were recorded in flat cells at room temperature using manganese oxide as an internal standard.

Distribution and metabolism of SA in mice

To determine the optimum dosage of ASA and the appropriate sampling time for *in vivo* experiments using mice, mice were administered 100–400 mg/kg of ASA dissolved in 0.5 M sodium bicarbonate intraperitoneally and were sacrificed at 10, 20 or 60 min after the injection. The serum, liver, colon, kidney, lung, spleen, thymus and brain were collected and SA and 2,3-DHBA were immediately analyzed by HPLC–ECD method.

Estimation of hydroxyl radical generation in multiple organs of mice after whole-body irradiation

The mice were administered with 400 mg/kg of ASA i.p. and whole-body X-ray irradiation was started at 9 min after the injection. At 20 min after the injection of ASA, the mice with or without irradiation were sacrificed and the samples were collected. The analyses of SA and 2,3-DHBA were performed as soon as possible after the sampling. To evaluate the hydroxyl radical generation, the ratios of 2,3-DHBA to SA were compared between the irradiated mice and the control mice. Moreover, to estimate the amount of increase in hydroxyl radicals corresponding to 2,3-DHBA in the samples, we derived two linear-regression equations from our *in vitro* experiments and *G*-value of hydroxyl radical (2.7/100 eV) by γ-radiolysis of water at neutral pH [19]. Using these linear-regression equations, the equivalent amounts of hydroxyl radicals in the samples were calculated as follows: (1) the expected values of hydroxyl radicals generated by 24 Gy irradiation were calculated from the concentration of SA in the samples, and (2) the equivalent amounts of hydroxyl radicals corresponding to the 2,3-DHBA increases in the samples were calculated by subtracting the background (un-irradiated samples) from the concentrations of 2,3-DHBA in the samples.

Statistical analysis

The regression equations, correlation coefficients and levels of significance were analyzed using SPSS analytical software, version 13. Data from studies with only two groups were analyzed by the Student *t*-test for equal variance or the Welch *t*-test for unequal variance after Bartlett's test.

Results and discussion

In the present study, to elucidate the relationship between salicylate hydroxylation and hydroxyl radical generation, we selected ionizing radiation (X-ray irradiation) with a low linear energy transfer (LET) as a possible source of hydroxyl radicals, because water molecules absorb energy from ionizing radiation and yield free radicals such as hydroxyl radical. In addition, low LET ionizing radiation generate hydroxyl radicals in line with the track and it is possible to perform uniform whole-body irradiation of

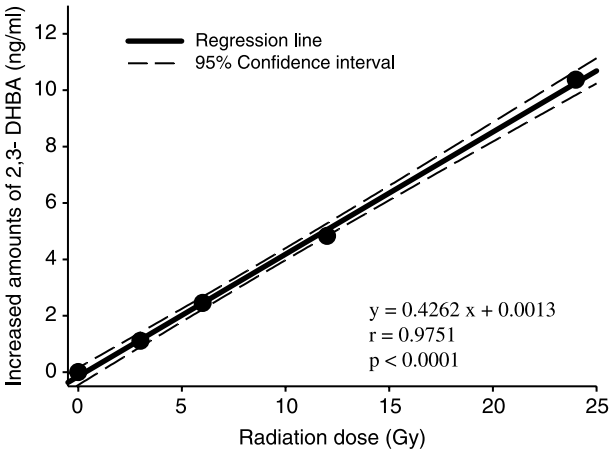

Figure 1. Scatter plots and regression analysis of increased amounts of 2,3-DHBA versus radiation dose *in vitro*. SA (100 μg/ml, pH 7.2) in a test tube was irradiated with X-rays (2.22 Gy/min, 3–24 Gy), and the concentrations of 2,3-DHBA were determined by the HPLC–ECD method. The regression line and the 95% confidence interval were calculated by SPSS analytical software.

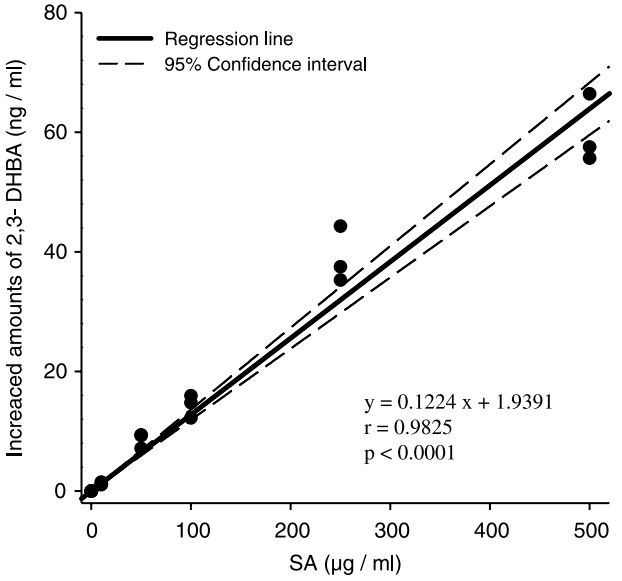

Figure 2. Scatter plots and regression analysis of increased amounts of 2,3-DHBA versus concentration of SA *in vitro*. SA (10–500 μg/ml, pH 7.2) in a test tube was irradiated with X-rays (2.22 Gy/min, 24 Gy), and the concentrations of 2,3-DHBA were determined by the HPLC–ECD method. The regression line and the 95% confidence interval were calculated by SPSS analytical software.

mice with these X-rays [19]. Moreover, it is easy to control hydroxyl radical generation by using the on–off switch of the X-ray generator.

Figure 1 presents the relationship between the increase in 2,3-DHBA and the radiation dose *in vitro*. When a solution of SA at a concentration of 100 μg/ml in 100 mM Tris–HCl buffer (pH 7.2) was irradiated with X-rays, significant correlations were observed between the amounts of increase in 2,3-DHDA (y; ng/ml) and radiation dose (x; Gy): $y = 0.4262x + 0.0013$, $r = 0.9751$, $p < 0.0001$. On the other hand, when SA was dissolved in diethylether instead of the buffer, there was no increase in the concentration of 2,3-DHBA after 24 Gy of X-ray irradiation (data not shown), indicating that hydroxyl radicals cannot be generated from diethylether by X-ray irradiation. These results strongly suggested that 2,3-DHBA was formed directly by the reaction of SA with hydroxyl radicals generated from H_2O exposed to X-rays *in vitro*.

As shown in Figure 2, the amounts of increase in 2,3-DHBA formed by irradiation with 24 Gy of X-rays was dependent on the concentration of SA (10–500 μg/ml). There were also significant correlations between the increase in 2,3-DHBA (y; ng/ml) by 24 Gy irradiation and the concentrations of SA in the test tubes (x; μg/ml). The following linear-regression equations were observed: $y = 0.1224x + 1.9391$, $r = 0.9825$, $p < 0.0001$. These data also showed that no more than about 0.02% of the total SA in the test tube was hydroxylated even by 24 Gy of X-rays.

Further, the generation of hydroxyl radicals under the same *in vitro* experimental conditions was examined by the ESR spin trapping method. As shown in Figure 3, the DMPO–OH adduct signal in the reaction mixtures was detected after the X-ray irradiation, and the relative intensity of the DMPO–OH adduct increased along with the radiation dose of X-ray (Figure 4). It had been reported that Tris had the ability to scavenge hydroxyl radical [20], however, we confirmed that we could detect the same spectra with similar intensity when we used 100 mM phosphate buffer, pH 7.2 instead of the Tris buffer.

From these *in vitro* results, we obtained the following the linear-regression equation in order to calculate the predictive increased amounts of 2,3-DHBA related to hydroxyl radical generation by 24 Gy of X-ray irradiation:

$$c = 0.1224x + 1.9391$$

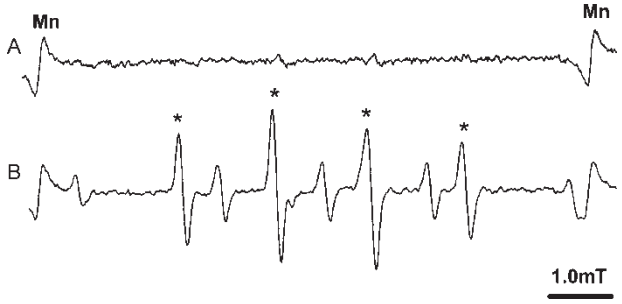

Figure 3. ESR spectra of DMPO–OH (labeled *) obtained from aqueous solution (100 mM Tris–HCl, pH 7.2) containing 200 mM DMPO before (A) or after (B) X-ray irradiation (2.22 Gy/min, 6 Gy) *in vitro*. ESR analysis was started at 1 min after the end of X-ray irradiation.

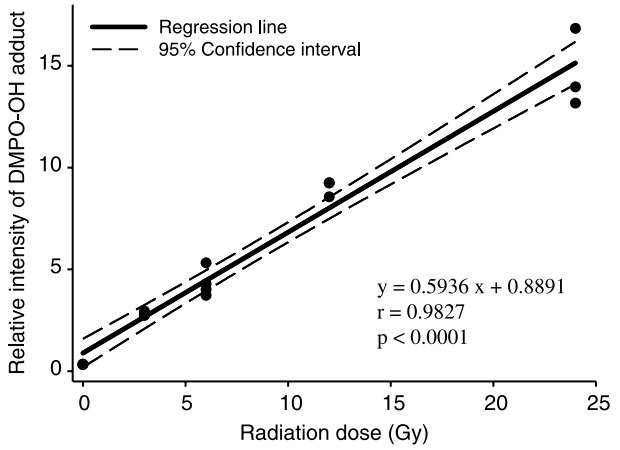

Figure 4. Relative intensity of DMPO–OH adducts and radiation does of X-ray *in vitro*. The regression line, correlation coefficient and critical rate were calculated by SPSS analytical software from the data of 0–24 Gy.

c, the predictive amounts of increase in 2,3-DHBA in the buffer after 24 Gy of irradiation (ng/ml); *x*, the concentration of SA in the buffer before the irradiation (μg/ml).

The tissues contains many hydroxyl radical scavengers, however, the reaction between hydroxyl radical scavenger and hydroxyl radical depends on the affinity of the scavenger to hydroxyl radical. Because of the high affinity of SA to hydroxyl radical, SA has been used as a useful probe for hydroxyl radical in many *in vitro* and *in vivo* experiments. Moreover, previous reports have shown that the administration of SA effectively prevented cellular damages caused by hydroxyl radical [21–23], suggesting that SA may act as an extreme effective hydroxyl radical scavenger *in vivo*. We also examined the formation of 2,3-DHBA from SA (100 μg/ml) in 10% liver or colon homogenate with 100 mM Tris–HCl buffer (pH 7.2) exposed to 24 Gy of X-rays at 25°C. The results showed that there was no statistically significant difference in the formation of 2,3-DHBA by the irradiation between the homogenates and the buffer alone (data not shown), implying that the formation of 2,3-DHBA by the reaction of SA with hydroxyl radical observed *in vitro* appears to be similar to that seen *in vivo*. Thus, we calculated the predictive increased values (*c*) of 2,3-DHBA produced by 24 Gy of X-ray *in vivo* according to the above *in vitro* regression equation, to determine the optimum dosage of ASA and the appropriate sampling time for *in vivo* experiments.

First at 1 h after the i.p. administration of 100 mg/kg ASA, the concentration of SA and 2,3-DHBA in the organs of mice were examined without X-ray irradiation, because all previous *in vivo* studies using the salicylate hydroxylation method examined the 2,3-DHBA levels at 1 h after the administration of 100 mg/kg of SA or ASA [2,4,5]. We could detect

Table I. Distribution of SA and the formation of 2,3-DHBA in the serum, liver and colon of mice at 1 h after ASA injection without irradiation and predictive values of 2,3-DHBA produced by 24 Gy of X-irradiation.

	SA (μg/ml or g)	2,3-DHBA (ng/ml or g)	Predictive value of 2,3-DHBA by irradiation (ng/ml or g)
Serum	99.5 ± 30.2	110.7 ± 35.3	13.89
Liver	11.4 ± 8.3	20.2 ± 10.9	3.03
Colon	18.8 ± 9.3	15.4 ± 5.0	3.94

Mice were injected intraperitoneally with ASA (100 mg/kg), and the samples were collected at 1 h after the injection without X-irradiation. The predictive values of 2,3-DHBA produced by 24 Gy of X-irradiation were calculated from the mean SA concentration by using a regression equation based on the *in vitro* experiments.

2,3-DHBA to some extent in the samples after SA administration without X-irradiation (Table I). On the other hand, the levels of 2,5-DHBA, the main metabolite of SA, were 5–10 fold higher than 2,3-DHBA (data not shown). We could not conclude the participation of enzymes or basal hydroxyl radical formation in these 2,3-DHBA elevations. As shown in Table I, when we calculated the predictive values (*c*) of 2,3-DHBA by 24 Gy of irradiation from the actual SA concentrations in the each mouse organ we found that they were too small to detect because the SD values of the 2,3-DHBA concentrations in serum and organs without irradiation were much bigger than the predictive values.

Thus, we repeated the experiment at a higher dosages of ASA (400 mg/kg i.p. injection), examined the levels of 2,3-DHBA at 10, 20 and 60 min after the injection, and again the predictive values (*c*) of 2,3-DHBA by 24 Gy irradiation were calculated from the actual SA concentrations in each organ (Table II). It is generally accepted that the predictive increased values (*c*) of 2,3-DHBA by 24 Gy irradiation can be detected with a success rate of greater than 95% when the sum of the predictive increased value (*c*) of 2,3-DHBA by 24 Gy irradiation and the mean 2,3-DHBA concentration in each organ is larger than the sum of the mean 2,3-DHBA concentration and twice the standard deviation (Table II). According to this guideline, by 20 min after the administration of ASA, a significant increase of 2,3-DHBA by the irradiation should be detectable in most organs such as serum, liver, colon, spleen, thymus and brain. Based on these results, we examined the levels of 2,3-DHBA in the organs of mice exposed to X-rays at 20 min after the i.p. injection of 400 mg/kg ASA.

As expected, there were detectable increases in the total levels of 2,3-DHBA by the 24 Gy X-ray irradiation in various tissues at 20 min after the ASA injection (Table III). However, it should be noted that the levels of SA in the organs were also altered by the

Table II. Distribution and metabolism of ASA in the serum or several organs of mice at 10 and 20 min after the injection without irradiation, and predictive values of 2,3-DHBA produced by X-irradiation.

	Minutes	SA (μg/ml or g)	2,3-DHBA (ng/ml or g)	Mean +2 SD	Predictive value by irradiation + mean of 2,3-DHBA (ng/ml or g)
Serum	10	322.9 ± 84.6	28.7 ± 2.1	32.9	41.5 + 28.7 = 70.2*
	20	448.3 ± 95.3	55.1 ± 3.6	62.3	56.8 + 55.1 = 111.9*
	60	324.6 ± 92.3	408.8 ± 201.6	812.0	41.7 + 408.8 = 450.5
Liver	10	75.0 ± 23.7	15.9 ± 5.9	27.7	11.1 + 15.9 = 27.0
	20	82.4 ± 1.5	42.9 ± 4.0	50.9	12.0 + 42.9 = 54.9*
	60	51.4 ± 12.9	106.5 ± 57.0	220.5	8.2 + 106.5 = 114.7
Colon	10	153.8 ± 52.8	13.5 ± 5.1	23.7	20.8 + 13.5 = 34.3*
	20	90.9 ± 12.1	17.3 ± 0.1	17.5	13.1 + 17.3 = 30.4*
	60	37.3 ± 20.9	51.2 ± 37.3	125.8	6.5 + 51.2 = 57.7
Kidney	10	95.4 ± 30.1	22.7 ± 6.7	36.1	13.6 + 22.7 = 36.3*
	20	155.3 ± 42.0	67.0 ± 18.1	103.2	20.9 + 67.0 = 87.9
Lung	10	90.1 ± 27.3	25.8 ± 9.7	45.2	13.0 + 25.8 = 38.8
	20	241.4 ± 0.6	81.2 ± 30.5	142.2	31.5 + 81.2 = 112.7
Spleen	10	84.9 ± 7.3	23.9 ± 5.6	35.1	12.3 + 23.9 = 36.2*
	20	128.4 ± 50.4	33.1 ± 2.9	38.9	17.7 + 33.1 = 50.8*
Thymus	10	63.7 ± 21.2	23.2 ± 11.6	46.4	9.7 + 23.2 = 32.9
	20	92.4 ± 1.8	27.3 ± 0.7	28.7	13.2 + 27.3 = 40.5*
Brain	10	16.8 ± 4.4	6.8 ± 4.4	15.6	4.0 + 6.8 = 10.8
	20	46.6 ± 4.4	18.8 ± 1.3	21.4	7.6 + 18.8 = 26.4*

Mice were injected intraperitoneally with ASA (400 mg/kg), and the samples were collected at 10 or 20 min after the injection without X-irradiation. The predictive values of 2,3-DHBA produced by 24 Gy of X-ray irradiation were calculated from the mean SA concentration by using a regression equation based on the *in vitro* experiments.

* Predictive value of 2,3-DHBA + the mean 2,3-DHBA concentration +2 SD

X-ray irradiation. With respect to SA levels, a previous study has also shown that differences in 2,3-DHBA levels among animals are closely associated with the levels of SA [24]. In addition, the present study showed that the increases in the amount of 2,3-DHBA were dependent on the concentration of SA even *in vitro* (Figure 2). Therefore, the present and previous study demonstrated that the ratios of 2,3-DHBA to SA might be useful as an indicator of hydroxyl radical generation *in vivo*. As shown in Table III, significant

Table III. Concentrations of SA and 2,3-DHBA in the serum or several organs at 20 min after the ASA administration with or without X-irradiation and the equivalent amounts of hydroxyl radicals for 2,3-DHBA increases in the samples.

		SA (μg/ml or g)	2,3-DHBA (ng/ml or g)	2,3-DHBA/SA ($\times 10^3$)	Equivalent amount of ·OH for 2,3-DHBA increase (nmol/ml or mg)
Serum	Control	371.7 ± 98.4	73.3 ± 20.5	0.196 ± 0.023	
	24 Gy	339.8 ± 56.8	94.3 ± 13.9	0.279 ± 0.028**	3.241
Liver	Control	77.9 ± 15.5	36.2 ± 5.5	0.455 ± 0.143	
	24 Gy	93.0 ± 10.8	38.1 ± 4.6	0.430 ± 0.062	
Colon	Control	89.3 ± 18.3	15.7 ± 3.2	0.236 ± 0.080	
	24 Gy	74.7 ± 17.0	27.9 ± 1.9	0.440 ± 0.091*	7.397
Kidney	Control	168.2 ± 64.8	38.2 ± 14.8	0.340 ± 0.101	
	24 Gy	73.3 ± 2.4	50.2 ± 14.5	0.688 ± 0.218*	7.390
Lung	Control	90.2 ± 40.3	32.7 ± 15.5	0.301 ± 0.039	
	24 Gy	109.7 ± 8.1	62.0 ± 21.8	0.623 ± 0.167*	12.812
Spleen	Control	118.2 ± 41.3	30.3 ± 3.8	0.292 ± 0.111	
	24 Gy	98.2 ± 31.3	64.5 ± 23.1	0.498 ± 0.142*	16.462
Thymus	Control	76.7 ± 2.8	23.4 ± 4.9	0.407 ± 0.152	
	24 Gy	99.0 ± 9.2	39.6 ± 9.7	0.379 ± 0.141	
Brain	Control	25.5 ± 8.3	14.2 ± 6.3	0.503 ± 0.246	
	24 Gy	29.8 ± 7.6	11.5 ± 2.6	0.420 ± 0.043	

Mice were injected intraperitoneally with ASA (400 mg/kg), and then received whole body irradiation with X-rays (24 Gy). The samples were collected at 20 min after the injection. The equivalent amounts of hydroxyl radicals for the increases in 2,3-DHBA in the samples by X-ray irradiation were calculated for each mean value of SA and 2,3-DHBA concentration by using the regression equations described in "Results and Discussion". *, ** Significant increase compared with the control without irradiation ($p < 0.05$ or 0.01).

increases in the ratio of 2,3-DHBA to SA were observed in serum and in most organs such as colon, kidney, lung and spleen of mice irradiated with X-rays, compared to the un-irradiated controls. These results strongly indicated that the salicylate hydroxylation method might be useful for determination of hydroxyl radical generation in various organs *in vivo*.

In contrast, there were no significant increases in these ratios in the liver, thymus and brain (Table III), suggesting that there was no increase of hydroxyl radicals in these organs of mice irradiated with X-rays. Since the SA concentrations in all of these organs were increased by the X-ray irradiation, it is possible that these organs have a greater ability to scavenge hydroxyl radicals than other organs, possibly through other antioxidants besides SA. Further study is needed to confirm this hypothesis.

Moreover, the large differences seen among the values of 2,3-DHBA/SA in the control organs without irradiation indicate that it is hard to compare hydroxyl radical generation quantitatively between organs using the ratios of 2,3-DHBA to SA alone, although this parameter is useful in the detection of hydroxyl radical generation *in vivo*. Accordingly, we attempted to calculate the amount of increase in hydroxyl radicals corresponding to the increases in 2,3-DHBA in the organs of whole-body irradiated mice in order to compare hydroxyl radical generation quantitatively among different organs. By using the above-mentioned regression equation ($c = 0.1224x + 1.9391$), we were able to calculate the predictive amounts of 2,3-DHBA (c; ng/g or ml) generated by 24 Gy of X-ray irradiation from the actual concentration of SA (x; μg/g or ml) in the sample, because there is a good correlation between the amount of increase in 2,3-DHBA and the amount of generated hydroxyl radicals if the concentrations of SA are constant. Further, the generated amounts of hydroxyl radicals in 1 ml of water (assuming specific gravity = 1) exposed to 24 Gy of low LET ionizing radiation is calculated at 6.719 nmol/ml or g from G-value of 2.7/100 eV at neutral pH [19]. Therefore, it is possible to estimate the amounts of hydroxyl radicals (y; nmol/g or ml) corresponding to the 2,3-DHBA increases by means of the following regression equation using the former predictive amounts of 2,3-DHBA (c) and the amounts of increase of 2,3-DHBA in the tissue (z; ng/g or ml).

$$y = 6.7192 \div c \times z$$

c, the predictive amounts of increase in 2,3-DHBA in the buffer after 24 Gy of irradiation (ng/ml); z, the increased amounts of 2,3-DHBA in the tissue (ng/g or ml).

As shown in Table III, the amounts of hydroxyl radicals corresponding to the 2,3-DHBA increases ranged from 3.241 to 16.462 nmol/ml or g in the samples where a significant increase in the ratio of 2,3-DHBA to SA had been detected. The theoretical amount of hydroxyl radicals generated by 24 Gy of X-irradiation from water is 6.7192 nmol/ml or g, and in fact the colon and kidney showed values similar to this theoretical value. It is extremely difficult to obtain a complete overview of hydroxyl radical generation in *in vivo* system because the metabolites of chemicals in the organs are continuously changing by metabolic turnover, and hydroxyl radical generation by low LET radiation is thought to be affected by circumstantial conditions, such as pH and N_2O concentration [19]. However, in order to compare the levels of hydroxyl radical generation among organs it was thought to be more helpful to use together with the ratio of 2,3-DHBA to SA. Previous studies which succeeded in detection of significant increase of 2,3-DHBA indicated that some chemicals could produce hydroxyl radicals more effectively than our X-ray irradiation experiment [17,18,25]. Therefore, equivalent values of hydroxyl radicals as calculated by our method might be a helpful parameter for estimating hydroxyl radical generation induced by chemicals as well as by irradiation *in vivo*.

Reactive oxygen species are produced by most cells under normal and pathological conditions, and among these, the highly reactive hydroxyl radical has been shown to induce lipid peroxidation, DNA and RNA damage, and mitochondrial disruptions, leading to cell death, carcinogenesis and so on. Although the *in vivo* measurement of hydroxyl radicals is extremely difficult, the present study demonstrated the usefulness of salicylate hydroxylation as a method for detection of hydroxyl radicals generated by X-rays in multiple organs of mice *in vivo*. However, before applying this method *in vivo*, it will be necessary to compare the levels of 2,3-DHBA in the organs of non-irradiated animals injected with ASA with the predictive levels after by X-ray irradiation, to determine the optimum dosage of ASA and the appropriate sampling time. The salicylate hydroxylation method may be useful in examining the involvement of hydroxyl radical in the cellular damages caused by X-rays as well as by chemical agents *in vivo*.

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