Automated analysis of the serum antioxidative activities against five different reactive oxygen species with the sequential injection and chemiluminescence detection methods

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Abstract

Background: There is a growing evidence that reactive oxygen species (ROS) may cause many pathologic conditions including chronic diseases, neurodegenerative disorders, cancer and aging. There are a number of methods to measure the total antioxidative activity of the serum. However, since the lifetime and oxidative activity of various types of ROS are all different, to measure simply the total antioxidative activity of the serum is not enough. Therefore, to aid the diagnosis and to improve the therapeutic strategy, it is important to develop a simple and reliable method of assaying antioxidative activity of the serum.

Methods: A method that combines sequential injection analysis (SIA) and luminol chemiluminescence (CL) detection was employed for the measurement of antioxidative activities of human serum. We collected sera from healthy subjects (n=42) and patients with diabetes (n=39) and rheumatoid arthritis (n=25) and tested the sensitivity, reproducibility and reliability of our method.

Results: Since the operation is automatically controlled by a personal computer, we obtained a satisfactory repeatability without the need of much manpower. The time required for obtaining the antioxidative activity against one ROS for one individual is less than 3 minutes. Although the value of antioxidative activity varies from subject to subject, there were a certain relationship between the disease and the antioxidative values of each type of ROS. The results suggest that the measurement of antioxidative activity against different ROS may provide us with valuable information regarding the disease state.

Conclusions: The evaluation of antioxidative activities against each ROS by the proposed method should be more informative to understand the antioxidative status of biological fluid.

Introduction

Reactive oxygen species (ROS) generated in biological systems are involved in signal regulation, production of energy, phagocytosis and defense mechanism against infection. However, the excess generation of ROS induces harmful biological oxidation. Indeed, ROS have been implicated in the pathophysiology of several conditions including diabetes, infertility, rheumatoid arthritis and cardiovascular diseases [1-4]. Much evidence indicates that exposure to ROS cause deleterious changes in cell function by a number of oxidative modification such as lipid peroxidation, enzyme inactivation and oxidative DNA damage, ultimately results in cell death [5-8]. In these aspects, the evaluation of the degrees of oxidative damage caused by ROS is essential in order to clarify the contribution of ROS to several diseases.

On the other hand, it is known that there are different kinds of ROS including superoxide anion (O_2^-), nitric oxide (NO), hydrogen peroxide (H_2O_2), hypochlorite anion (ClO^-) and singlet oxygen ($^1O_2$). Since each ROS has different chemical features including lifetime and oxidative activity, the oxidative damage on the living body and resulting disease should be different. Therefore, it is important to evaluate the degrees of oxidative damage due to each ROS in order to understand the relationship between ROS generation and pathogenesis.

In this study, we focused on the endogenous antioxidative activity of human serum as an indicator of oxidative damage due to each ROS. It is known that human serum possess antioxidants and antioxidative enzymes to protect the body against oxidation by ROS. The concentrations and/or activities of them should decrease by the scavenging

of excessively generated ROS in the body of patients.   As a result, the antioxidative activity of serum of patients might be lower than that of healthy human.

The task of quantifying antioxidative activity of biological samples can be approached in three different ways.   Firstly, the concentrations of all of the individual molecules that are currently recognized as antioxidants are measured.   However, it does not account for the influence of undiscovered antioxidants or the substances that are technically difficult to be assayed.   Secondly, the antioxidative activity can be measured by the decreasing ratio of 1,1-diphenyl-2-picrylhydrazyl radical or peroxy radical scavenging activities [9,10].   However, these methods can be applied only for specific radicals.   Thirdly, ferric reducing ability of plasma method [11] and total radical-trapping potential method [12] are famous for assay of antioxidative activity of biological sample; however, they are based on the total reduction ability of samples. Since ROS differ from each other with respect to chemical features including reactivity and life-time, antioxidative activities of biological samples against each ROS should be different.   However, as mentioned above, all the assays for the total antioxidative activity of biological samples cannot meet the requirement.   Therefore, the method for determination of antioxidative activities against each ROS will be required.

In our laboratory, antioxidative activity measurement method for plant extracts has been developed, based on batch method with luminol chemiluminescence (CL) detection [13].   Luminol emits light when it reacts with ROS.   In this method, the attenuation of luminol CL due to scavenging of ROS by sample was measured as the antioxidative activity.   Successively, luminol CL detection was hyphenated with

sequential injection analysis (SIA) for determination of antioxidative activities against ClO$^-$, $^1O_2$, O_2^- and NO [14-16].   Employing SIA makes it possible to reduce sample volume, reagent consumption and human error and obtain repeatable results, comparing with flow injection analysis [17].

In this study, we exploit the SIA-CL method to automated analysis of the serum antioxidative activity against five different ROS.   The method has an advantage for determination of antioxidative activities against each ROS because different ROS can be generated by altering the reagents in the SIA system.   The developed method was applied to the serum samples collected from healthy subjects, diabetic and rheumatoid patients and the measured antioxidative activities were compared.

Material and methods

Reagents and solution

Hypoxanthine (HX), L-ascorbic acid, boric acid, sodium hydroxide, H_2O_2 and sodium acetate were purchased from Wako Pure Chemical (Osaka, Japan); xanthine oxidase (XOD), *N*, *N*-dimethylformamide (DMF), luminol and sodium hypochlorite were from Nacalai Tesque (Kyoto, Japan); (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR1) was from Dojindo (Kumamoto, Japan); *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES), lactoperoxidase (LPO) and sodium bromide were from Sigma (St. Louis, MO, USA).   Water was deionized using an Autostill WG 220 (Yamato Kagaku, Tokyo, Japan) and passed through a Puric-Z (Organo, Tokyo, Japan).   All other solvents and

reagents were of analytical grade.   Luminol was dissolved in DMF and then diluted with water (DMF content was 1%).   The reagents used for generation of each ROS are summarized in Table 1.

Apparatus and manifold design

The SIA-CL system (Fig. 1) consisted of a Cavro XL 3000 syringe pump (volume 1 mL, Cavro Scientific Instruments Inc., CA, USA), a multiport valve (six-port, Cavro Smart Valve), a CL detector (CLD-10A, Shimadzu, Kyoto, Japan) and a recorder (RIKADENKI R-01, Tokyo).   A CL reagent (luminol solution) was pumped by an LC-10ADvp (Shimadzu).   The syringe pump and multiport valve were computer-controlled using Pump: Link Evaluation Software (Cavro).   Table 2 shows the sequence of operations program for the control of the SIA-CL system.   The appropriately defined volumes of reagents and sample were sequentially aspirated into a holding coil (1 m x 0.5 mm i.d., PTFE tube).   The reagents and sample zones were then propelled toward the CL detector.   The CL reagent was 200 mM luminol dissolved in 1% DMF aqueous solution.   The CL reagent was introduced to the stream just before the CL detector at a flow-rate of 1.0 ml/min, and the peak height of the resulting CL signal was recorded.   Each measurement was repeated in triplicate and the mean of peak height values were used in the evaluation of the experiments.   All measurements were carried out at ambient temperature (24 ± 4 °C).

Measurement of antioxidative activity

Ten μL of the human serum was diluted with phosphate buffer saline (PBS) by 300, 100, 20, 20 and 10 times for the measurement of antioxidative activity against ClO^-, H_2O_2, $^1O_2$, O_2^- and NO, respectively.   And then, 5 μL of the diluted serum was injected into the SIA system.   The injected serum volume corresponded to 16.7 nl for ClO^-, 50 nl for H_2O_2, 250 nl for O_2^- and $^1O_2$ and 500 nl for NO, respectively.   Antioxidative activity was calculated by the following equation:

$$\text{Antioxidative activity } (\%) = (CLI_B - CLI_S)/CLI_B \times 100$$

Where CLI_B and CLI_S represent the CL intensities obtained from blank (PBS) and serum sample, respectively.

Sample collection

Serum samples were obtained from 42 healthy volunteers (17 females, 25 males; age range, 33-62 years; mean age 48.9±7.5) and from 39 diabetic patients (21 females, 18 males; age range, 39-83 years; mean age 64.8±11.9) and 25 rheumatic patients (21 females, 4 males; age range, 31-84 years; mean age 61.2±12.7) attending Sasebo Chuo Hospital.   The glycosylated hemoglobin values of diabetic patients and the C-reactive protein values of rheumatic patients were significantly higher than those of healthy subjects.   The collected serum samples were frozen at -80°C prior to analysis. Experiments were performed according to Helsinki Declaration.   All subjects gave their informed consent to participate in this study, which was approved by the Ethics Committee of Graduate School of Biomedical Sciences, Nagasaki University.

Data analysis

The data are presented as mean ± standard error (SE) for the number of experiments. The current study employed the decision tree method proposed by Kobayashi [18,19]. Bartlett's test is used as a test for the homogeneity of k variance. Then, if the k sampled populations have equal variances (p>0.05 by the Bartlett's test), Dunnett's multiple comparison test is performed; otherwise, Steel's test is used. All the statistical tests were two-sided at a significant level of α=0.05.

Results and discussion

At first, for each ROS, we determined the dynamic range of the serum volume for the antioxidative activity. A linear relationship was observed when the logarithm of the serum volume was plotted against the antioxidative activity because the effect of serum volume on antioxidative activity showed sigmoidal curve. Based on the linear range, the injection volume of serum was set by diluting it with PBS.

Repeatability of signal responses on SIA-CL analysis of human serum

Figure 2 shows typical CL responses when the antioxidative activity of human serum against $^1O_2$ was measured by the SIA-CL system. When the volume of the serum was increased, the corresponding CL response derived from $^1O_2$ decreased. Hence, it was thought that antioxidative components in human serum scavenged $^1O_2$. The decreased amount of the CL should correspond to the total antioxidative activity of the serum components against $^1O_2$. The relative standard deviations (RSD) of the within

– (n = 3) and between – day (n = 3) repeatabilities of the antioxidative activities of serum from healthy subjects against each ROS were less than 4.9 and 11.8%, respectively. On the other hand, the RSD of the within – (n = 5) and between – day (n = 3) repeatabilities of the blank CL signals were less than 6.5 and 8.6%, respectively.

Antioxidative activity of serum of diabetic and rheumatic patients

The measurement results of antioxidative activities of the serum samples are summarized in Table 3. All the tested samples exhibited antioxidative activity against 5 types of ROS. We compared the antioxidative activities of serum samples between healthy subjects and patients. Serum from diabetic patients exhibited a significantly lower antioxidative activity against NO, $^1O_2$ and O_2^- than that from healthy subjects while the activity against ClO^- and H_2O_2 increased, comparing with healthy subjects. Also, the antioxidative activity of serum against H_2O_2 from rheumatic patients was higher than that from healthy subjects. The proposed SIA-CL method can grasp the difference of antioxidative activities between healthy subjects and ROS-mediated diseases. Since ROS are involved in the pathogenesis of diabetes and rheumatoid arthritis [1,3] and a part of antioxidants in the serum are consumed, a decrease in the antioxidative activity of the serum from the patients was expected, comparing with healthy subjects. However, the antioxidative activities against some ROS increased in patients, contrary to the expectation

The increased activity against H_2O_2 in diabetic patients may be due to the low H_2O_2 production. Alba-Loureiro *et al.* reported that H_2O_2 production was decreased in

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impaired neutrophils from diabetic rats [20]. They found that the activities of three enzymes involved in the metabolism of glucose and glutamine were changed and concluded that this change might play an important role in the impaired neutrophil function observed in diabetes. Also, it was described that the phorbol myristate acetate-stimulated neutrophils from normal volunteers generated approximately 4-fold increases in H_2O_2 compared with these from diabetic patient by Inoue *et al.* [21]. Since the decrease in H_2O_2 production naturally leads to decrease in ClO^-, the antioxidative activity against ClO^- might increase in diabetic patients. Our results were supported by these observations.

In the comparison of healthy subjects and rheumatic patients, the antioxidative activities against H_2O_2 significantly increased in rheumatic patients compared with healthy subjects. It was reported that the activity of catalase, which quenches H_2O_2, was increased by inflammation [22]. The increase of catalase activity may increase the antioxidative activity in patients.

Redox mechanisms have been implicated in the pathogenesis of numerous human diseases, either by direct ROS-mediated tissue damage, or via gene transcription induced through redox sensitive transcription factors. Consequently, there have been increasing interest in measuring the antioxidative activity of biological fluids and tissues and a number of different assays of total antioxidative activity have been developed [23-32]. However, the results obtained with this study indicated that the antioxidative activity of biological fluids fluctuates for two reasons. Namely, (i) because of different types of ROS, and (ii) in accordance with the disease state. Therefore, the

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measurement of only the total antioxidative activity may mislead the diagnosis.   As we reported in this paper, the antioxidative activity against each ROS should be separately measured.   Such results would be very valuable, because they could provide us valuable information.

In conclusion, we developed a method that combined SIA and luminol CL detection for the measurement of antioxidative activity against five types of ROS in the human serum.   We tested the method by using the sera obtained from healthy subjects and patients.   The result clearly indicated that this equipment would be a valuable tool for research, diagnosis and therapy.

List of Abbreviations

CL   chemiluminescence

ClO^- hypochlorite anion

DMF   *N*, *N*-dimethylformamide

HEPES   *N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulfonic acid

H_2O_2 hydrogen peroxide

HX   Hypoxanthine

LPO   lactoperoxidase

NO   nitric oxide

NOR1 $(\pm)$-(E)-4-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide

O_2^- superoxide anion

$^1O_2$ singlet oxygen

PBS   phosphate buffer saline

ROS   reactive oxygen species

RSD   relative standard deviation

SE   standard error

SIA   sequential injection analysis

XOD   xanthine oxidase

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Figure captions

Fig. 1 Scheme of the SIA-CL system for the measurement of antioxidative activity of human serum against each ROS.   SP, syringe pump; HC, holding coil; MV, multiport valve; M, mixing tee; P, pump: D, detector; R, recorder.

Fig. 2 Signal responses of scavenging capacity against $^1O_2$ for human serum.   Five µL of PBS (blank) and serum diluted 40 (125 nl), 20 (250 nl) and 10 times (500 nl) with PBS were injected into the SIA system.
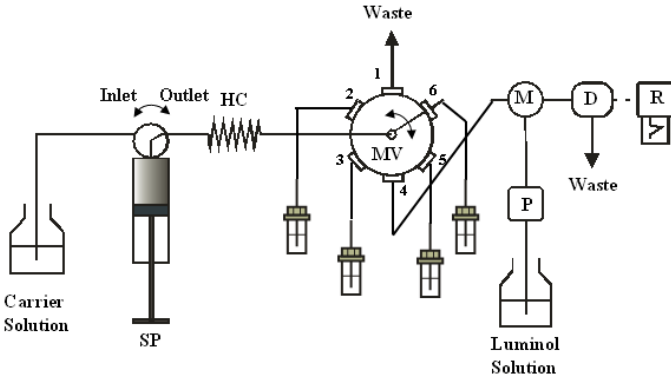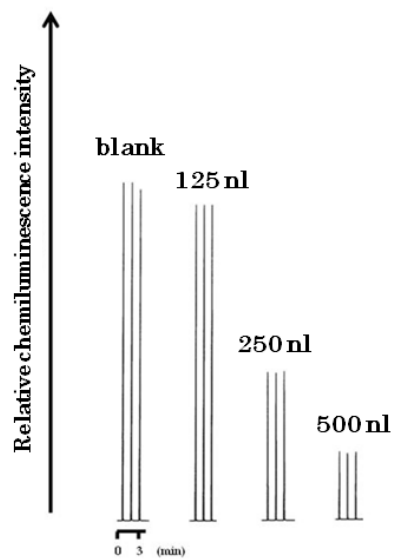
Fig. 1

Fig. 2

Table 1   Carrier solution and ROS generation reagent for the SIA-CL measurement

ROS	Carrier solution	ROS reagent
ClO^-	50 mM borate buffer (pH 9.5)	38.3 mM NaClO/carrier solution
NO	100 mM Hepes buffer (pH 8.2)	2 mM NOR1/DMSO:100 mM HCl = 1:1
$^1O_2$	100mM sodium acetate buffer (pH 4.5)	150 mM H_2O_2 /carrier solution
		80 mg/ml LPO/carrier solution
		250 mM NaBr/carrier solution
O_2^-	100 mM Hepes buffer (pH 9.5)	1.6 units/ml XOD/carrier solution
		1.0 mM HX/carrier solution
H_2O_2	50 mM borate buffer (pH 7.4)	100 mM H_2O_2/carrier solution

Table 2 Operation program of the SIA-CL measurement

ROS	Step	Event	Valve position of SP	Valve position of MV	Flow rate (ml/min)	Volume (µl)
ClO^-	1	Aspiration of carrier solution	Inlet	-	1.5	600
	2	Aspiration of NaClO	Outlet	2	0.4	20
	3	Aspiration of sample or blank	Outlet	6	0.4	5
	4	Propulsion to CL detector	Outlet	4	0.8	625
NO	1	Aspiration of carrier solution	Inlet	-	1.5	600
	2	Aspiration of sample or blank	Outlet	6	0.4	5
	3	Aspiration of NOR1	Outlet	2	0.4	5
	4	Propulsion to CL detector	Outlet	4	1.5	610
$^1O_2$	1	Aspiration of carrier solution	Inlet	-	1.5	600
	2	Aspiration of sample or blank	Outlet	6	0.4	5
	3	Aspiration of H_2O_2	Outlet	3	0.4	10
	4	Aspiration of LPO	Outlet	5	0.4	5
	5	Aspiration of NaBr	Outlet	2	0.4	5
	6	Propulsion to CL detector	Outlet	4	1.5	625
O_2^-	1	Aspiration of carrier solution	Inlet	-	1.5	600
	2	Aspiration of sample or blank	Outlet	6	0.4	5
	3	Aspiration of XOD	Outlet	2	0.4	10
	4	Aspiration of HX	Outlet	5	0.4	10
	5	Propulsion to CL detector	Outlet	4	2	625
H_2O_2	1	Aspiration of carrier solution	Inlet	-	1.5	600
	2	Aspiration of H_2O_2	Outlet	2	0.4	20
	3	Aspiration of sample or blank	Outlet	6	0.4	5
	4	Propulsion to CL detector	Outlet	4	0.8	625

Table 3 Antioxidative activity in the serum from healthy subjects (n=42) ant patients with diabetes (DM, n=39) and rheumatoid arthritis (RA, n=25)

		Antioxidative activity			
		Range (%)	Mean ± SE[b] (%)	95% CI[c] (%)	Statistical test
ClO^-	Healthy	46.3-83.4	62.2 ± 1.7	58.7-65.6	-
	DM	51.7-75.8	65.5 ± 0.9	63.7-67.3	healthy < DM [a]*
	RA	52.6-74.5	63.0 ± 1.0	60.9-65.1	-
NO	Healthy	31.7-89.6	70.7 ± 1.6	67.4-74.0	-
	DM	5.7-95.7	62.7 ± 3.0	56.7-68.7	healthy > DM [a]**
	RA	47.1-79.4	69.3 ± 1.5	66.1-72.4	
$^1O_2$	Healthy	52.2-77.9	66.8 ± 1.1	64.6-68.9	-
	DM	33.3-80.0	55.1 ± 2.0	51.1-59.1	healthy > DM [a]**
	RA	54.3-83.2	69.4 ± 1.3	66.7-72.0	-
O_2^-	Healthy	3.0-21.4	11.4 ± 0.6	10.2-12.6	-
	DM	-5.8-15.7	6.6 ± 0.8	5.0-8.3	healthy > DM [a]**
	RA	3.1-23.5	12.5 ± 1.2	10.0-14.9	-
H_2O_2	Healthy	10.7-23.2	18.9 ± 0.4	18.0-19.7	-
	DM	23.5-35.6	30.0 ± 0.5	29.1-31.0	healthy < DM [a]**
	RA	18.9-25.6	22.4 ± 0.3	21.7-23.1	healthy < RA [a]**

a) Steel's test

b) Standard error

c) Confidence interval

*$p<0.05$

**$p<0.01$