Original Article

UVB-dependent generation of reactive oxygen species by catalase and IgG under UVB light: Inhibition by antioxidants and anti-inflammatory drugs

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ABSTRACT: Catalase, which can decompose H_2O_2, has recently been found to generate unspecified reactive oxygen species (ROS) as a result of ultraviolet B (UVB) irradiation. Many proteins, hemes, and iron compounds were first tested to determine that this ROS generation was unique to catalase and immunoglobulin G (IgG). An increase in absorbance at 502 nm due to 2',7'-dichlorofluorescein, the oxidized product of 2',7'-dichlorodihydrofluorecein diacetate as a result of UVB (310 nm) irradiation, was measured in order to estimate this ROS generation. Catalase and IgG generated a pronounced amount of ROS when irradiated with UVB. Another heme protein, cytochrome c, and heat-inactivated catalase had no such effect. ROS generation by catalase was at least 5 times more potent than that reported for IgG with UVB and without antigens. This catalase-mediated ROS generation was largely temperature-dependent in the range of 25 to 42°C. As IgG is considered an evolutionally important bactericidal component, the same was considered true for this enzyme. Next, inhibitory effects of various drugs, including antioxidants and anti-inflammatory drugs, on catalase-mediated and UVB-induced ROS generation were examined. Many of the drugs, including catalase inhibitors, had inhibitory effects with different potencies. Melanin was found to be the most effective inhibitor of this ROS generation (IC_{30}, 0.2 μg/mL), followed by Indigo Carmine and rutin. Also inhibiting this ROS generation were ascorbic acid, α-tocopherol, indomethacin, coenzyme Q10, β-carotene, uric acid, piroxicam, diclofenac, and glutathione, in that order of potency. Various ROS were apparently generated by catalase under UVB, creating a cycle or chain which was thought due to the biphasic effects of some drugs such as 3-aminotriazole or sodium azide. Excess ROS generation induces inflammation. Catalase might serve dual roles, removing H_2O_2 and generating various ROS depending on the H_2O_2 concentration and other factors.

Keywords: Catalase, IgG, UVB, Reactive oxygen species (ROS), Antioxidant, Anti-inflammatory drug, Melanin

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Introduction

This study primarily sought to verify and expand upon two recent reports (*1,2*) that found unexpected generation of reactive oxygen species (ROS) as a result of ultraviolet (UV) light irradiation of catalase and immunoglobulins. Heck *et al.* (*1*) found a material in keratinocyte lysates capable of generating ROS responding to UVB (290-320 nm) irradiation. They purified this material into a protein of 240 kDa and verified that it was catalase using antibodies; this finding was surprising as this enzyme is the only one to degrade H_2O_2, a typical ROS that can be scavenged by catalase and glutathione peroxidase (GSH•Px). The current study attempted to identify the type of ROS generated by UVB and catalase and indicate which drugs can inhibit the generation of this ROS. The study measured the fluorescence (495 nm excitation and 520 nm emission) due to 2',7'-dichlorofluorescein (DCF), the oxidized product of 2',7'--dichlorodihydrofluorecein

diacetate (DCFH•DA) (*1*). Valkonen *et al*. (*3*) noted that DCFH can also be assayed by spectrophotometry as an increase in optical density (O.D.) at 504 nm; therefore, this method of spectrophotometry was used in the current study to facilitate testing the effects of numerous drugs. Another fascinating finding is that of Wentworth, Jr. *et al*. (*2*), who reported that UVB (312 nm) irradiation of immunoglobulins (IgG, IgM, IgA) and T-cell receptor resulted in ROS generation from water. The inter-Greek key domain interface (IGKD), formed by heavy and light chains of the IgG molecule, is considered to be the site of H_2O_2 generation from singlet oxygen ($^1O_2$) or hydrogen trioxide (HOOOH) generated by UV light (*4*). As $^1O_2$ has a short life span (4 μs), it must be converted into H_2O_2 to maintain continuous microbicidal action for the immune system. $^1O_2$ can also be formed from hypochlorite (HOCl) plus H_2O_2 and superoxide radical (O_2^-) plus nitric oxide (*5*). This means that the generation of $^1O_2$ is not limited to UV irradiation. In addition to $^1O_2$, ozone (O_3) was reported to be involved in leukocyte antibacterial action (*6*). Contrary to the generally accepted concept that $^1O_2$ is an end-product ROS, antibodies have an intrinsic ability to generate $^1O_2$ in the first step of ROS generation. The strongest oxidant is the hydroxyl radical (•OH), which can be produced by ferrous ion from H_2O_2; thus, the participation of •OH could not be ignored in the current experiments. *In vivo*, the main sources of $^1O_2$ and H_2O_2 generation are NAD(P)H oxidase in leucocytes and xanthine oxidase in organs such as the liver and intestines. However, the participation of these enzymes should be excluded in this *in vitro* report. The current authors previously reported that UV irradiation (260-340 nm) for 60 min generated H_2O_2 from 100 μg/mL IgG that was inhibited by dexamethasone and piroxicam (*7*). This suggested that anti-inflammatory drugs could block the ROS (H_2O_2 or its derived oxidants). The current study found that catalase's ROS-generating capacity under UV light was more evident than that of IgG. None of the other proteins examined was capable of generating more ROS than catalase or IgG. The nature of ROS generated by catalase under UVB light was further investigated using various medications including catalase inhibitors, antioxidants, and anti-inflammatory drugs.

Materials and Methods

Materials

2',7'-dichloro-5-fluorecein diacetate (DCFH•DA) and Arabia gum were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Catalase (from bovine liver, approx. activity = 50,000 units/mg/protein, 2 × crystallized), Cu, Zn-superoxide dismutase (Cu, Zn-SOD, from bovine erythrocytes, lyophilized powder, approx. activity = 3,000 unit/mg/protein), and glutathione peroxidase (GSH•Px, from bovine erythrocytes, lyophilized powder containing approx. 20% protein) were from Sigma Co. (St. Louis, USA). Polyclonal anti-human immunoglobulin G (IgG from fraction powder from rabbit, H&L, 9.7 mg/mL) was obtained from Inter-Cell Technologies, Inc. (New Jersey, USA). Dexamethasone acetate (Decadron-A 8 mg/mL) was from Banyu Pharmaceutical Co. (Tokyo, Japan). Desferoxamine mesylate (Desferal) was purchased from Ciba-Geigy Co. (Takarazuka, Japan). All other reagents were from Nakalai Tesque Co. (Kyoto, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Sigma Co. (St. Louis, USA).

UVB irradiation

Mixed with 1.5 mL of water were 0.3 mL phosphate buffer (1 M, pH 7.4) and 0.3 mL catalase (250 μM), followed by the addition of 0.3 mL Arabia gum suspension (100 mg/mL) with or without a drug. A fresh DCFH•DA solution (15 mM, 0.6 mL) was added to a total volume of 3.0 mL. An aliquot (0.3 mL) was transferred to a quartz cell containing 2.7 mL water maintained at 37°C. Absorption at 502 nm was measured (value A_0) by a spectrophotometer (Shimadzu UV-1200, Japan). The resulting mixture (2.7 mL) was irradiated with UVB (310 nm) for 15 min. An aliquot (0.3 mL) of this UVB-irradiated mixture was added to another quartz cell containing 2.7 mL water kept at 37°C. The absorbance was next measured at 502 nm to yield an A_{15} value. The irradiation measurement slit was 5 mm × 10 mm (50 mm^2), and the cell surface area 300 mm^2; therefore, the sample received one-sixth of the irradiation. The beam power was 0.012 μW/mm^2 at 310 nm (as measured by the manufacturer) so that 0.002 μW/mm^2 (= 2 μW/cm^2) was irradiated for 15 min (in general), resulting in 30 mJ/cm^2. Measurement was performed at various irradiation (210-650 nm) and absorption (480-520 nm) wavelengths. Irradiation time-dependency for an increase in 502 nm absorption was confirmed over 60 min.

Measurement of DCFH•DA oxidation

In this report, the increase in O.D. at 502 nm due to DCF (an oxidative product of DCFH•DA) was generally measured following UV (310 nm) irradiation for 15 min at 37°C. Various compounds including catalase, IgG, and cytochrome c were assayed for their ability to increase O.D. at 502 nm as a result of irradiation. The scavengers of $^1O_2$, O_3, *etc*. and various kinds of drugs were tested to ascertain the nature of catalase-generated ROS under UVB irradiation. Drug inhibition was calculated by the following formula:

$$\text{Inhibition \%} = \left[1 - \frac{\text{Drug } (A_{15}-A_0)}{\text{Control } (A_{15}-A_0)}\right] \times 100$$

where A_0 and A_{15} are the absorbances at 502 nm before and after, respectively, irradiation at 310 nm.

Arabic gum was used to dissolve drugs in a final concentration of 10 mg/mL. The concentration needed to obtain 30% inhibition (IC_{30}) was estimated from drug concentration-% inhibition curves as an index of the drug's inhibitory potency.

Results

ROS generated by catalase with light of different wavelengths

As expected, the maximum wavelength at which ROS were generated by catalase (25 μM) was 310 nm (UVB) (Figure 1). However, 210 and 280 nm (UVC) for 15 min also increased ROS, as did 350 nm (UVA). Visible blue light (450 nm) resulted in a 77% increase over the generation at 310 nm. Even red light (650 nm) generated about half the ROS generated at 310 nm. Spectra from DCF were observed between 480 and 520 nm (data not shown). Optimal O.D. was between 503 and 510 nm. A sharp decrease occurred near 504 nm, which has been adopted for assay by other investigators (*1*), so 502 nm was chosen for measurement of ROS generation in the current study. Nadirs of spectra were also seen at 486, 493-497, and 510-528 nm.

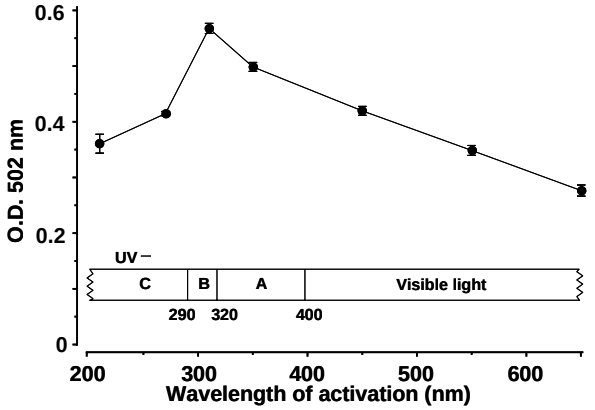

Figure 1. Effect of irradiation on catalase-mediated ROS generation. DCFH•DA solution (3 mM, pH 7.4) was irradiated for 30 min at 37°C in the presence of 25 μM catalase. The increase in absorbance at 502 nm (O.D. at 502 nm) was measured. Bars indicate S.E. of the mean (n = 3).

ROS generation by various proteins, iron compounds, and dyes

To determine whether only catalase generated ROS, other compounds were investigated. Concentrations of the compounds were set at 10 mg/mL; the corresponding calculated molar concentrations are noted in Table 1. Catalase generated the largest quantity of ROS, followed by IgG. However, heat-inactivated catalase lacked the capacity to generate ROS. Cu, Zn-SOD, bovine serum albumin (BSA), myoglobin, and $FeSO_4$ also enhanced ROS generation in high concentrations. Mn-SOD, monoamine oxidase, RNase A, lysozyme and ubiquitin failed to generate ROS. No noticeable ROS generation was observed with L-histidine. Some amino acids are reported to scavenge rather than generate $^1O_2$ and O_2^- like, for example, L-histidine and L-tryptophan, respectively (*10,11*). Rose Bengal (*12*) and methylene blue (*13*), photosensitizers that generate $^1O_2$, did not increase O.D. at 502 nm under the experimental conditions, suggesting that catalase generated ROS in a different way than these dyes did. Since catalase is an enzyme that contains heme, some hemes and inorganic irons were also then examined. Hemes (hematin and hemin) and heme-containing proteins (cytochrome c and myoglobin) did not generate ROS in the current system. Hematoporphyrin, which does not possess

Table 1. ROS generated by UVB irradiation in the presence of various proteins, iron compounds, and dyes

Agent (10 mg/mL)	Conc. (M)	ΔO.D. at 502 nm ($\times 10^{-3}$)[a]
Catalase	4.2×10^{-5}	489 ± 28
Inactivated Catalase	4.2×10^{-5}	26 ± 3
Immunoglobulin G (IgG)	1.5×10^{-5}	253 ± 24
Cytochrome C	8.0×10^{-4}	33 ± 11
Cu, Zn-SOD	3.2×10^{-4}	124 ± 29
Mn-SOD	2.5×10^{-4}	89 ± 31
Bovine serum albumin (BSA)	1.5×10^{-4}	127 ± 9
Monoamineoxidase (MAO)	3.8×10^{-5}	91 ± 17
Myoglobin	5.9×10^{-4}	156 ± 16
Hematin	1.6×10^{-2}	9 ± 6
Hemin·Cl	1.5×10^{-2}	12 ± 8
$FeSO_4 \cdot 7H_2O$	3.6×10^{-2}	136 ± 43
$FeCl_3 \cdot 6H_2O$	4.0×10^{-2}	4 ± 3
Hematoporphyrin·2HCl	1.4×10^{-2}	13 ± 2
Rose Bengal	9.8×10^{-3}	23 ± 8
Methylene blue	2.7×10^{-2}	17 ± 6
RNase	7.1×10^{-4}	46 ± 38
Lysozyme	6.9×10^{-4}	4 ± 3
Ubiquitin	1.2×10^{-3}	13 ± 8
L-Histidine·H_2O	4.8×10^{-2}	11 ± 6

[a] Absorbance increase at 502 nm was measured after 15 min of UVB (310 nm) irradiation with DCFH•DA (3 mM) at 37°C, pH 7.4. Molar concentration was calculated from each reported molecular weight.

iron, also failed to generate ROS. Inorganic ferrous iron ($FeSO_4$) generated a small amount of ROS, albeit only in high concentrations, but ferric iron ($FeCl_3$) generated none whatsoever. Thus, the possibility that trace iron contaminating the tested proteins was a ROS-generating component can be negated. Some conformational structure(s) in catalase and IgG may be responsible for generating the unspecified but obvious ROS observed in this experiment.

The concentration-dependency of three representative compounds is shown in Figure 2. Almost linear concentration-dependent increases of O.D. at 502 nm (ROS generation) were observed with catalase. Catalase is clearly shown to be the best mediator to generate ROS, followed by IgG. Cytochrome c was ineffective. Other compounds that were tested in various concentrations showed much less ROS generation than did IgG (data not shown).

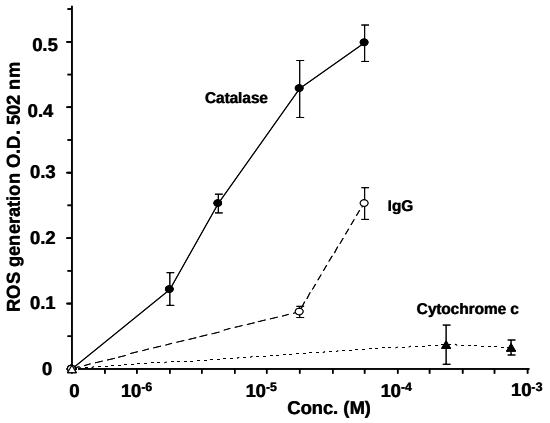

Figure 2. ROS generation mediated by catalase (●), IgG (○) or cytochrome (▲) when irradiated with UVB (310 nm) for 15 min at 37°C. Bars indicate S.E. of the mean (n = 3).

Catalase- and DCFH•DA-concentration dependency of ROS generation

DCFH•DA concentration dependency was confirmed up to 4 mM, with 10, 25 and 50 μM of catalase (Figure 3). Therefore, inhibition of ROS generation by drugs was examined using 3 mM DCFH•DA and 25 μM catalase. The time course of this ROS generation was linear up to 30 min of irradiation (data not shown), so a UV irradiation time of 15 min was chosen to evaluate a drug's inhibitory effect.

Temperature-, pH- and phosphate buffer concentration-dependency of ROS generation by catalase as a result of UVB irradiation

ROS generation was temperature-dependent (Figure 4). Drug inhibition was tested at 37°C with 25 μM catalase, which had a sufficient increase in O.D. at 502 nm. Five different pHs of 100 μM potassium phosphate buffer were selected. ROS generation (O.D. at 502 nm) of 0.512, 0.502, and 0.510 (n = 3) was almost equal at pH 6.8, 7.4, and 8.0, respectively. ROS generation of 0.419 and 0.346 was lower at pH 5.6 and at pH 8.6, respectively. When phosphate buffer was replaced with water, ROS generation was 0.422 (n = 3). At potassium phosphate concentrations of 50, 100, and 200 mM (pH 7.4) ROS generation was 0.532, 0.502 and 0.411, respectively (n = 3). Hence, phosphate was not essential for ROS generation.

Effect of moderately soluble or suspended drugs

ROS generation (O.D. at 502 nm, 0.469, n = 3) significantly increased in the presence of dimethyl formamide (DMF) (0.5-2.0%, Figure 5A). The DMF concentration required to solubilize water-insoluble drugs in a final concentration of 100 μg/mL was above

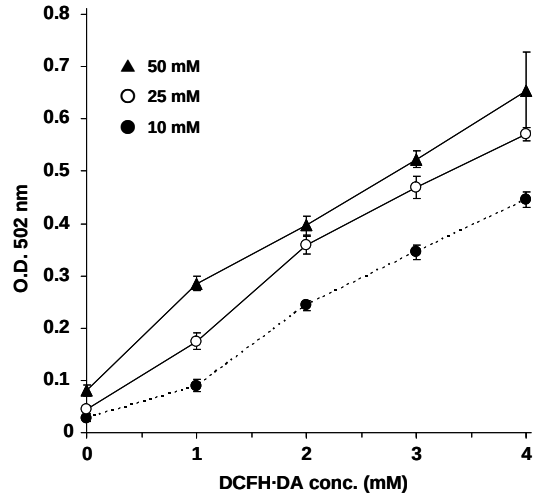

Figure 3. The concentration dependency of catalase-mediated and UVB-induced ROS generation. DCFH•DA (0-4 mM) and catalase (10-50 μM) solutions (pH 7.4) were irradiated with UVB for 15 min at 37°C.

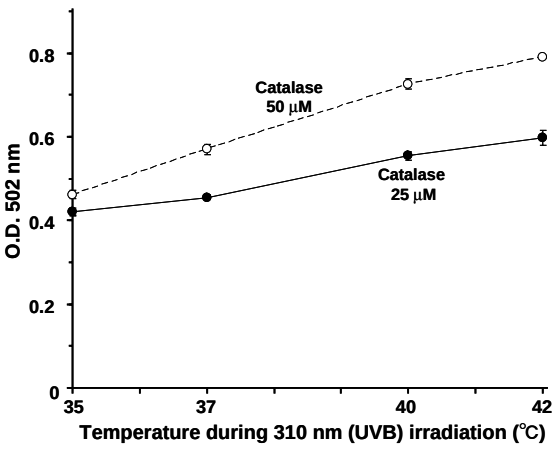

Figure 4. The temperature dependency of catalase-mediated and UVB-induced ROS generation. A 50 μM (○) or 25 μM (●) catalase-DCFH•DA (3 mM) solution (pH 7.4) was irradiated with UVB for 15 min at 37°C. Bars indicate S.E. of the mean (n = 3).

1.0%. This concentration of DMF alone markedly increased O.D. at 502 nm by approximately 45% (Figure 5A). Dimethyl sulfoxide (DMSO) alone resulted in a similar enhancement of O.D. at 502 nm (data not shown). On the other hand, Arabia gum (5-20 mg/mL) slightly decreased ROS generation (Figure 5B) and did not precipitate the drugs. In addition, 5, 10, and 20 mg/mL of Arabic gum did not affect the inhibitory potencies on ROS generation of Indigo Carmine, ascorbic acid, rutin, and indomethacin (data not shown). Therefore, 10 mg/mL Arabia gum, which only inhibited ROS generation by 11%, was selected because of its suitability in homogeneously suspending different kinds of drugs.

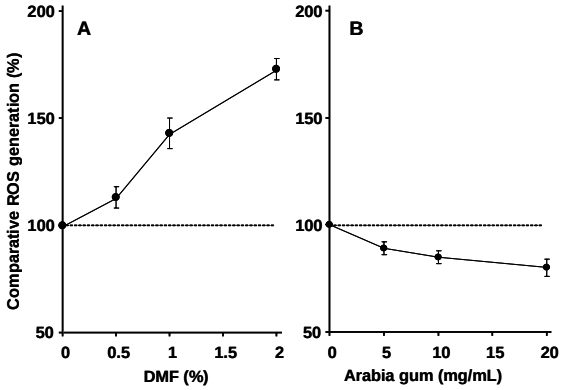

Figure 5. Effects of dimethyl formamide (DMF) (A) and Arabic gum (B) on catalase-mediated and UVB-induced ROS generation. Bars indicate S.E. of the mean ($n = 3$).

Drug inhibition of ROS generation

In the current assay system, drugs could have reacted directly with DCFH•DA, without catalase or UVB participation, resulting in an artificial increase in absorption at 502 nm. Therefore, a continuous curve at 502 nm was registered for up to 15 min without catalase or UVB light in a separate experiment. There was neither a remarkable increase nor decrease in absorption with any drug without catalase. When catalase was absent, some drugs increased absorption before the start (A_0) and maintained the same O.D. until the end of irradiation (data not shown). Figure 6 shows the inhibitory effects up to 100 μg/mL of 12

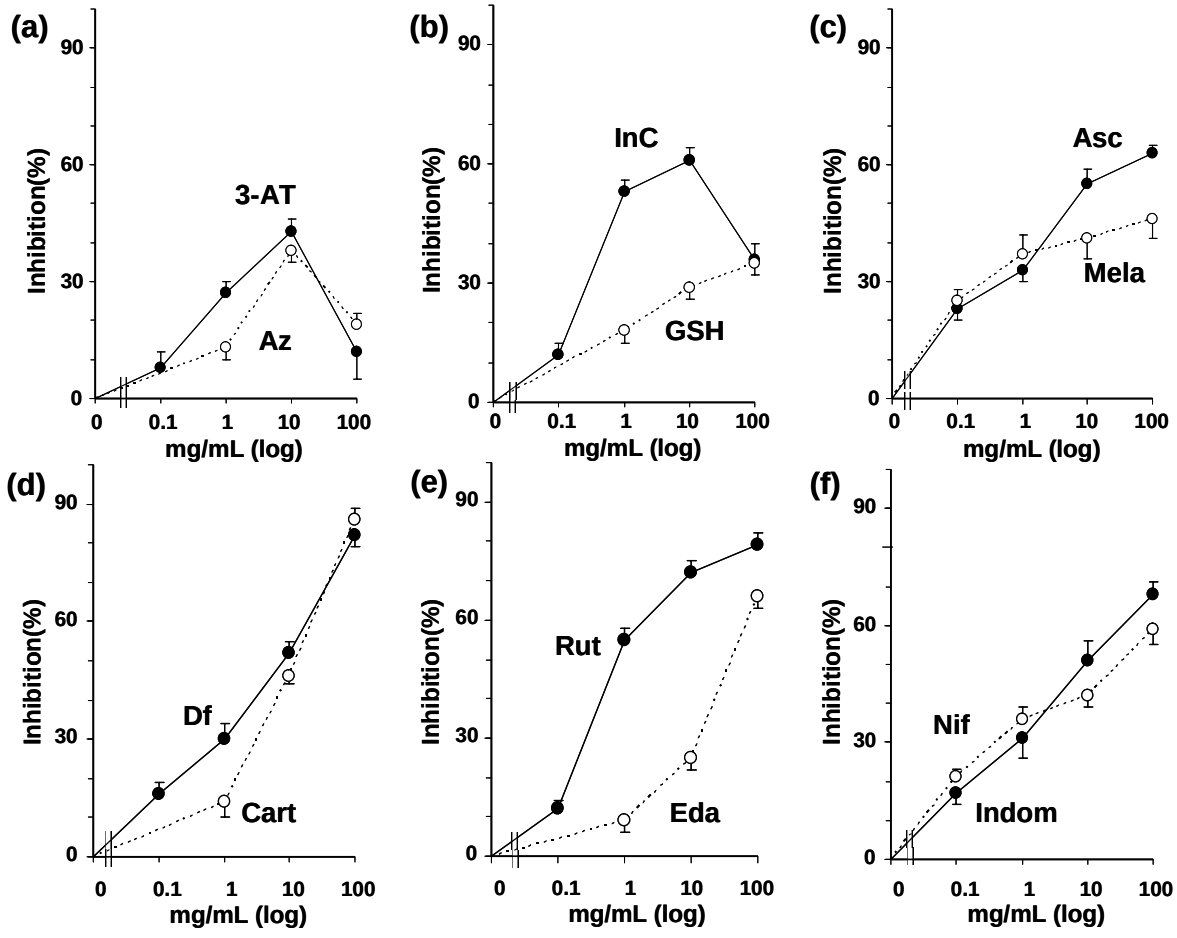

Figure 6. Inhibitory effects of various drugs on ROS generation induced by catalase under UVB irradiation. Drugs were suspended in 100 mM phosphate buffered solution (pH 7.4) containing 10 mg/mL Arabic gum, 25 μM catalase, and 3 mM DCFH•DA. Irradiation with UVB was carried out for 15 min at 37°C. 3-AT, 3-aminotriazole; Az, azide; InC, Indigocarmin; GSH, reduced glutathione; Asc, ascorbic acid; Mela, melanin; Df, diphenylisobenzofuran; Cart, β-carotene; Rut, rutin; Eda, edaravone; Nif, nifedipine; Indom, indomethacin. Bars indicate S.E. of the mean ($n = 3$).

Table 2. Summary of inhibitory effects of various drugs on catalase-mediated and UVB-induced ROS generation[a]

IC_{30} < 1 μg/mL	1 < IC_{30} < 10 μg/mL	10 < IC_{30} < 100 μg/mL
Melanin (0.2)	Diphenylisobenzofuran (2)*	GSH (12)
Indigo carmine (0.3)*	Coenzyme Q10 (2)	Edaravone (12)
Rutin (0.3)	β-Carotene (3)	Nimesulide (15)
Ascorbic acid (0.6)	Uric acid (3)	Cis-Pt (30)
α-Tocopherol (0.8)	Piroxicam (3)	NADH (100)
Indomethacin (0.8)	Diclofenac (3)	
	Oxyphenbutazone (3)	
	Ibuprofen (7)	
	Aspirin (8)*	
	Azide (8)*	
1000 μg/mL < IC_{30} (Weak or No-inhibitory drug)		
Cu, Zn-SOD, GSH•Px*, Mannitol, DMTU, Desferal, BHT, Propyl gallate, Fenofibrate, Dexamethasone, Verapamil, DetaNONOate		

[a] IC_{30} (30% inhibitory concentration) was obtained from the inhibition curve of the drug. IC_{30} is indicated as μg/mL in parentheses. Drugs that showed biphasic inhibition where inhibitions were lower at high concentrations are marked with an asterisk (*).

drugs that appeared to have physiological significance. To compare potencies, drug concentrations here are expressed in μg/mL. A catalase inhibitor, 3-amino-1,2,4 triazole (3-AT), had biphasic inhibition reaching a peak of 43% at 10 μg/mL. Heck *et al*. (*1*) reported 45% and 25% stimulation at 1 mM (84 μg/mL) and 0.1 mM (8.4 μg/mL), respectively. However, they apparently neglected the inhibitory effect below 0.1 mM, and the current data showed no stimulatory effect over 100 μg/mL (1.2 mM). The same inhibitor, 3-AT, was also tested at 1,000 μg/mL and was found to stimulate 35% of the ROS generation occurring with catalase and UVB (data not shown).

A similar biphasic inhibition resulted with another catalase inhibitor, sodium azide (Figure 6a). Indigo Carmine, which is believed to quench O_3, inhibited ROS generation, resulting in 53% and 63% generation at 1 and 10 μg/mL (= 0.021 mM), respectively. Its dose-inhibition curve was also bell-shaped (Figure 6b). Its ROS generation was 36% at 100 μg/mL. The ROS observed here might include ozone, even if its existence is just transient. Reduced glutathione (GSH) inhibited ROS generation, resulting in 35% generation at 100 μg/mL (= 0.33 mM) (Figure 6b). A general antioxidant, ascorbic acid, inhibited ROS in the current system in a dose-dependent manner, and melanin (a skin product induced by sunlight and a polymer of tyrosine) inhibited ROS more potently (Figure 6c). The $^1O_2$ scavenger diphenylisobenzofuran (*14*) was a strong inhibitor of ROS generation (Figure 6d); thus, $^1O_2$ may also participate in the generation of ROS in the current system. Beta-carotene is a strong $^1O_2$ scavenger (*11*), and its inhibition was also strong (Figure 6d). Rutin scavenges various ROS and was found to inhibit ROS effectively in the current system (Figure 6e). Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a radical scavenger that is generally believed to quench •OH radicals but may also react with other ROS, was effective especially in higher concentrations (Figure 6e). Both indomethacin, a non-steroidal anti-inflammatory (NSAID), and nifedipine (Nif, a Ca-entry blocker and anti-inflammatory drug) showed strong inhibitory effects, with similar dose-dependent inhibition curves (Figure 6f).

The IC_{30} values of the drugs determined are listed in Table 2. The strong inhibitory effects on ROS generation in the current system were detected as an IC_{30} of less than 1 μg/mL for five drugs, *i.e*. melanin, rutin, ascorbic acid, α-tocopherol, and indomethacine. Ten medications, including several other antioxidants and NSAIDs, had moderate suppressive effects with an IC_{30} of less than 10 μg/mL, followed by GSH and four other drugs with an IC_{30} of less than 100 μg/mL. In contrast, deta NONOate, a NO donor, and dexamethasone had no effect, showing that NO and steroidals may not inhibit ROS generated by catalase under UVB irradiation. Two antioxidants (BHT, propyl gallate) also had no capacity for suppressing ROS generation. An anti-lipidemic drug, fenofibrate, was not effective, along with verapamil, a non-dihydropyridine Ca-entry blocker. Some important drugs, marked with an asterisk in the table, had biphasic inhibition, the mechanism and significance of which have yet to be clarified.

Discussion

Catalase catalyzes H_2O_2 and ROOH to ($H_2O + O_2$) and (H_2O + ROH), respectively, in a reaction that consists of two steps mediated by the compound I (catalase-[O]). An assay of catalase solutions that are pure enough to yield negligible absorption at 230 to 250 nm can be performed spectrophotometrically (*15*). Catalase works as a tetramer of the same or almost the same subunit, and enzymatic activity can be maintained only when all subunits are active (*16*). The reason for this is unclear. Catalase can remove high concentrations of H_2O_2, in contrast to glutathione peroxidase (GSH•Px), which is believed to play a main role in eliminating low concentrations of H_2O_2 *in vivo*. Nevertheless, catalase contains one hard-bound NADPH per subunit that offers a reductive power to GSH•Px; therefore, this

function of catalase cannot be ignored because of its importance at removing H_2O_2 *in vivo*. In fact, catalase has been reported to act as more of a primary defense against oxidative stress from H_2O_2 and UVB light than GSH•Px in human fibroblasts (*17*). UV light evidently generates ROS, but the species are unclear. Singlet oxygen is the most noted ROS generated by UV light (*18*); yet, at the same time $^1O_2$ also inactivates catalase (*19*). The discovery by Heck *et al*. (*1*) that catalase generates ROS despite its widely believed ability to reduce ROS (H_2O_2) was the first indication that catalase both removes and generates ROS. These contrasting reactions may occur depending on the concentrations of H_2O_2 and other ROS such as $^1O_2$. Recently, ROS generation by UV light and immunoglobulins has been highlighted (*2*). Immunoglobulins are reported to be antibacterial without binding to antigens. This concept could be enlarged to encompass catalase because this enzyme showed more ROS generation than IgG and both proteins specifically generated ROS under UVB (310 nm) irradiation (Figure 2). In terms of evolution, catalase is older than immunoglobulins if one is to speculate on the significance of a general host defense. However, catalase and IgG, and only high concentrations of Cu, Zn-SOD, BSA, myoglobin and FeSO4, generated ROS among the 20 compounds tested here (Table 1); catalase is worthy of consideration as an important specific source of ROS generation under UV light.

Because heat-inactivated catalase was inactive, a special peptide conformation of catalase appears to be important for ROS generation. Iron-containing cytochrome c, hematin, and hemin did not generate ROS. Ferrous iron, but not ferric iron itself, might increase this ROS generation slightly (Table 1). ROS generation was rather dependent on temperature, with generation occurring from 35-42°C (Figure 4). Catalase decreases in tumors (*20*), which might result in less in situ ROS generation by this enzyme, thus permitting tumor growth. Hyperthermia cancer therapy, which is generally carried out at 42°C, may possibly involve the effect of this increased ROS generation.

To ascertain whether UV light is specifically required for catalase to generate ROS, a separate experiment was performed in which UV irradiation was replaced with a typical radical initiator, 2,2'-azobis-2-1-amidinopropane (APPH) (*2*). This reagent works on unsaturated fatty acids, forming hydroperoxide (LOOH), malondialdehyde, or alkoxyl radical (LO•), but failed to generate ROS by reacting with catalase. AAPH (0, 50, 100 mM) with 25 μM catalase resulted in nearly the same absorption increase (0.56-0.60) (data not shown). Increased catalase (50 μM) also showed no difference, suggesting that AAPH had no effect on catalase-mediated ROS generation. Therefore, UV or visible light is essential for this ROS generation by catalase. However, what ROS are generated by UV light and catalase is unknown. A DCFH•DA oxidation method was used here to compare data with that from Heck *et al*. (*1*); this reagent is the best ROS detector but is not specific to a particular ROS. DCFH•DA does not detect H_2O_2 and lipid peroxides well because these reactions are very slow (*21*). DCFH•DA can be transformed to DCFH, which is oxidized by ROS to DCF. Here, DCF formed from DCFH•DA *in vitro* as a result of its reacting with mainly the •OH, peroxynitrite ($ONOO^-$), and the peroxyl radical (ROO•) must have been measured. Hypochlorite (OCl^-), $^1O_2$, O_2^-, and NO are weak at oxidizing DCFH to DCF. Less participation of $^1O_2$ in the oxidization of DCFH is reported (*22*). Bovine erythrocytes, Cu, Zn-SOD, and bacterial Mn-SOD did not generate much ROS under UV light (Table 1), even though Cu, Zn-SOD (SOD-1) have been reported to form •OH through co-existing H_2O_2 (*23*). Only catalase generated ROS under UVB irradiation; Cu, Zn-SOD, Mn-SOD and GSH•Px did not. DCFH•DA must indeed react with all ROS, but with different levels of reactivity. A catalase-UVB system might generate various ROS in numerous steps, creating chains or cycles among the ROS. If electron spin resonance (ESR) with a proper radical trap is used instead of DCFH•DA, O_2^- or •OH can be more specifically detected. However, their half-lives are limited and their roles in biological materials are not ROS-specific. In fact, there is little meaning in detecting these ROS to ascertain the role of catalase-generated ROS, and ESR methods cannot detect $^1O_2$, O_3, and H_2O_2. For now, the DCFH•DA method seems to be the best detector as it reacts with all kinds of ROS. Two •OH scavengers failed to inhibit catalase-generated ROS; therefore, •OH might not be one of the first ROS generated by catalase-UVB. ROS such as $^1O_2$ or O_3 could be among the first ROS generated and then be transformed to O_2^-, •OH, or the like. The increase of O.D. at 502 nm would be the sum of DCF formed by all of the ROS.

Effects of various ROS scavengers or antioxidants were examined in the system with 25 μM catalase under UVB (310 nm) irradiation. Catalase inhibitors 3-AT and sodium azide showed optimal inhibition of about 40% at 10 μg/mL (Figure 6a), but at higher concentrations stimulated ROS generation, as noted in a previous report (*1*). Therefore, the inhibitors had bell-shape (inhibited and increased) ROS generation. The H_2O_2, O_2^-, and •OH ratios may depend on the H_2O_2-decomposing capacity of catalase. Another possibility is unknown ROS generated in a milieu of 3-AT or sodium azide. •OH scavengers L-mannitol and dimethylthiourea (DMTU) showed neither inhibitory nor stimulatory effects on catalase's ROS generation (Table 2). In addition, the inhibition of ROS generation by Edaravone, noted to be a •OH scavenger (*24*), was not very potent especially in the lower concentration range. These results suggest that •OH might not be a major

ROS which is induced by catalase under UVB light. Determining any of the ROS from water induced by catalase and UVB as the main species might be difficult. On the other hand, Indigo Carmine, known to be a O_3 quencher (*6*), potently inhibited ROS generation but in a biphasic manner, suggesting that O_3 is one of the reactive species generated by catalase. The natural and intrinsic antioxidants ascorbic acid and α-tocopherol, which are reported to inhibit UV-induced human skin inflammation (*25*), effectively inhibited ROS generation in a dose-dependent manner (Table 2 and Figure 6). Melanin, a polymer of tyrosine that is produced in the skin by sunlight, was also found to be one of the most effective inhibitors among the drugs tested. Melanin may protect the skin against damage by UV light at least partly through its inhibitory effect on catalase-induced ROS generation, possibly in combination with antioxidative vitamins. Diphenylisobenzofuran and β-carotene are quenchers rather specific to $^1O_2$ and inhibited catalase-generated ROS (Figure 6d); though was less sensitive to DCFH•DA. Sodium azide is also known to be an effective scavenger of $^1O_2$ as well as a catalase inhibitor. $^1O_2$ should be considered a major ROS produced by catalase under UVB light.

The current authors previously reported (*7*) that IgG generated ROS under UV (280 nm) light irradiation for one hour. Interferon-γ and tumor necrotizing factor α, but not Cu, Zn-SOD, BSA, and histone, also increased absorption at 340 nm due to NADH oxidation as a result of UV irradiation, indicating ROS generation. ROS generation mediated by IgG was inhibited by dexamethasone, piroxicam, and dichlofenac but not by indomethacin (*7*). In this work, the NSAIDs tested, including piroxicam and dicrofenac, were found to be effective in the inhibition of catalase-induced ROS generation with UVB (310 nm) irradiation although their potencies differed. Indomethacin showed the strongest inhibitory effect and, in contrast, the steroidal dexamethasone had no effect (Table 2). Taken together, both catalase and IgG can generate ROS under UV light but probably *via* different mechanisms. The dihydropyridine Ca-entry blocker nifedipine, an anti-inflammatory, was a comparatively strong inhibitor of ROS generation (Figure 6f), while a non-dihydropyridine Ca-blocker, verapamil, showed no effect (Table 2). It appears that NSAIDs may generally inhibit ROS generation by catalase. NSAIDs are known to lower $^1O_2$ and H_2O_2 *via* the inhibition of NAD(P)H oxidase by macrophages (*8*) and leukocytes (*9*). The inhibitory effects of NSAIDs on catalase-induced ROS might need to be taken into account when considering their anti-inflammatory effects *in vivo*. The current results for antioxidants and anti-inflammatory drugs appear to be supported by several reports. Sulindac protected UVB-induced phototoxicity in the skin of SKH-1 hairless mice (*26*). Para-aminobenzoic acid scavenged ROS and protected DNA against UV and free radical damage *in vitro* (*18*). Salicylic acid protected catalase inactivation by damaging or substituting catalase-bound NADPH (*27*). Thus, investigating the relationship between catalase's ability to degrade H_2O_2 and generate ROS with other drugs in the future may prove interesting; this is especially true since the two contradictory actions might be influenced by factors such as the concentration of H_2O_2, the pH, and the lipophilicity of drugs. The site of initial ROS generation, which was reported to lie at the IGKD site for IgG (*4*), is likely to be important. IgG-UV-generated ROS are reported to be bactericidal without binding to antigens. The bactericidal effect of leucocytes may also be due to O_3 generated by antibodies (*28*). The current data is not sufficient grounds for speculation that catalase plays an important role in bactericidal or tumoricidal functions in skin or other organs; however, catalase's efficiency at generating ROS (five times that of IgG, Table 1) cannot be ignored. Catalase is, like IgG, also considered an evolutionarily important non-specific host defense tool.

In conclusion, catalase was found to be a unique protein that not only can decompose ROS (H_2O_2) but also efficiently generate ROS when specific energy such as UVB irradiation is supplied, although the major reactive species it generated have yet to be determined. ROS generation can be inhibited by numerous anti-inflammatory and antioxidative drugs. Natural antioxidants such as ascorbic acid, α-tocopherol, and melanin might play an important role in preventing damage due to possible catalase-induced ROS generation *in vivo*, and especially in the skin.

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