

# Antioxidant properties of aspirin: Characterization of the ability of aspirin to inhibit silica-induced lipid peroxidation, DNA damage, NF- $\kappa$ B activation, and TNF- $\alpha$ production

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## Abstract

Electron spin resonance (ESR) was used to investigate the reaction of aspirin toward reactive oxygen species, such as hydroxyl radicals ( $\cdot\text{OH}$ ), superoxide radicals ( $\text{O}_2^-$ ) and  $\text{H}_2\text{O}_2$ . The Fenton reaction ( $\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \cdot\text{OH} + \text{OR}$ ) was used as a source of  $\cdot\text{OH}$  radicals. The results show that aspirin is an efficient  $\cdot\text{OH}$  radical scavenger with a reaction rate constant of  $k = 3.6 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ , which is faster than several well established antioxidants, such as ascorbate, glutathione and cysteine. However, aspirin is not a good scavenger for  $\text{O}_2^-$  or  $\text{H}_2\text{O}_2$ . Through its antioxidant property, aspirin exhibited a protective effect against silica-induced lipid peroxidation and DNA strand breakage. Aspirin also inhibited the activation of nuclear transcription factor- $\kappa\text{B}$  induced by silica, lipopolysaccharide or the transition metal, Fe(II), as demonstrated by electrophoretic mobility shift assay. The results show that aspirin functions as an antioxidant via its ability to scavenge  $\cdot\text{OH}$  radicals. This antioxidant property may explain some of its various physiological and pharmacological actions. (*Mol Cell Biochem* **199**: 93–102, 1999)

**Key words:** aspirin, antioxidant properties, silica, lipid peroxidation, DNA damage, NF- $\kappa$ B, TNF- $\alpha$

## Introduction

Aspirin is a widely prescribed drug used primarily to treat inflammation. Long term use of aspirin in humans has also been reported to protect against the development of colon cancer and other digestive system cancers, including cancers of the esophagus and stomach [1–4]. Animal studies have shown that aspirin is able to inhibit chemically induced tumors of the colon, tongue, esophagus, pancreas, bladder, breast, liver, and skin, as well as various sarcomas [5–13]. Although extensive studies have been carried out, the mechanism of aspirin's action remains to be elucidated.

Recent studies have indicated that because the chemical structure of aspirin is similar to that of salicylic acid, aspirin may protect biological targets from hydroxyl radical ( $\cdot\text{OH}$ )-induced cellular injury by scavenging this radical [4–16].

It is generally believed that a delicate balance between intracellular oxidants and antioxidants influences biological functions [17–24]. Oxygen free radicals and their metabolites, collectively called reactive oxygen species, can induce cell injury, which may trigger a cascade of radical reactions promoting the disease process [17–26]. Biological systems are equipped with various antioxidants. If the balance

between oxidants and antioxidants shifts toward to oxidants, oxidative stress occurs [17–24]. Superoxide dismutase, catalase, ascorbate, and glutathione are examples of cellular antioxidants. Although aspirin may scavenge  $\bullet\text{OH}$  radical and inhibit this radical induced cellular injury, its antioxidant properties have not been properly characterized. For example, what is the reaction rate of aspirin with hydroxyl radical ( $\bullet\text{OH}$ ), the most reactive free radical among reactive oxygen species. Without the reaction rate, it is hard, if not impossible, to compare the  $\bullet\text{OH}$  radical scavenging property of aspirin to that of other scavengers, such as ascorbate and glutathione. In the present study, the reaction rate of aspirin with  $\bullet\text{OH}$  radicals was studied using an electron spin resonance (ESR) spin trapping technique. The Fenton reaction ( $\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \bullet\text{OH} + \text{OH}^-$ ) was used as a source of  $\bullet\text{OH}$  radicals.

In the present study, the effect of aspirin on silica-induced lipid peroxidation and DNA strand breakage was investigated. The effects of aspirin on nuclear transition factor (NF)- $\kappa\text{B}$  activation induced by silica, lipopolysaccharide (LPS) or transition metal,  $\text{Fe(II)}$ , and the production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) induced by silica were also investigated. These investigations were undertaken for two reasons. First, to further demonstrate the antioxidant properties of aspirin. Second, to elucidate the role of oxidant generation in silica-induced pulmonary reactions. Epidemiologic and pathologic studies have established that inhalation of silica incites the development of silicosis [27] and cancer [28]. Although the mechanisms of silica-induced lung injury remain to be investigated, recent studies indicate that  $\bullet\text{OH}$  radical generated by silica may play an important role [29, 30]. Through free radical reactions, silica particles cause DNA strand breaks, nuclear transcription factor activation, and tumor necrosis factor (TNF)- $\alpha$  production, which are considered to be involved in silica-induced fibrogenicity and carcinogenicity.

The major questions to be answered in this study are as follows: (1) Is aspirin a good  $\bullet\text{OH}$  radical scavenger? (2) If so, what is the reaction rate constant? (3) How does aspirin compare with other well established antioxidants, such as ascorbate? (4) Is aspirin a good scavenger for superoxide radicals ( $\text{O}_2^-$ ) or  $\text{H}_2\text{O}_2$ ? (5) Does aspirin inhibit silica-induced lipid peroxidation, DNA strand breaks, nuclear transcription factor NF- $\kappa\text{B}$  activation or TNF- $\alpha$  production? (6) Does aspirin inhibit NF- $\kappa\text{B}$  activation induced by LPS or  $\text{Fe(II)}$ , which are known to cause activation of this transcription factor via induction of oxidative stress?

## Materials and methods

### Reagents

Iron chloride ( $\text{FeCl}_2$ ),  $\text{H}_2\text{O}_2$ , 5,5-dimethyl-1-pyrroline N-oxide (DMPO), sodium formate, xanthine, xanthine oxidase, and

aspirin were purchased from Sigma Chemical Company, St. Louis, MO, USA. Chelex-100 chelating resin was purchased from Bio-Rad Laboratories, Richmond, CA, USA. Phosphate buffer (pH 7.4) was treated with chelex 100 to remove putative metal ion contaminants. The DMPO solutions were purified using activated Charcoal until free radical impurities disappeared as verified by ESR spectroscopy. Crystalline silica was obtained from U.S. Silica Products, Berkeley Springs, WV, USA and fractionated to  $<5\ \mu\text{m}$  using a Donaldson particle classifier. X-ray energy spectrometric measurements show that 98.7% of the particles were crystalline silica.

### Free radical measurements

ESR spin trapping [31, 32] was used to detect short-lived free radical intermediates. This technique involves an addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be studied by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splittings of the spin adduct are generally characteristic of the original, short-lived, trapped radicals. This method is specific and sensitive and is considered to be appropriate for detection and identification of free radical generation. All ESR measurements were made using a Varian E-9 spectrometer and a flat cell assembly. Hyperfine splittings were measured (to 0.1 G) directly from magnetic field separations using potassium tetraperoxochromate ( $\text{K}_3\text{CrO}_8$ ) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as standards. The relative radical concentration was estimated by multiplying half of the peak height by  $(\Delta H_{\text{pp}})^2$ , where  $\Delta H_{\text{pp}}$  represents peak-to-peak width. An EPR DAP 2.0 program was used for data acquisition and analysis. Reactants were mixed in test tubes in a total volume of 0.5 ml. The reaction mixture was then transferred to a flat cell for ESR measurement.

### $\text{H}_2\text{O}_2$ measurements

$\text{H}_2\text{O}_2$  was monitored by measuring the change in fluorescence of scopoletin in the presence of horseradish peroxidase. Fluorescence was monitored at an excitation wavelength of 350 nm and an emission wavelength of 460 nm using a Perkin-Elmer fluorescence spectrophotometer (model MPG-36).

### Lipid peroxidation measurements

Lipid peroxidation of the model polyunsaturated lipid, linoleic acid, by silica was measured by monitoring the thiobarbituric acid (TBA) reactive substances formed in the reaction mixture. A typical reaction mixture contained 10 mg/ml quartz particles

and 100  $\mu$ l of 0.9 M linoleic acid emulsion in a total volume of 1.0 ml HEPES buffered medium containing 140 mM NaCl, 5 mM KCl, and 10 mM HEPES (pH 7.4). The mixture was incubated for 1 h in a shaking water bath at 37°C. The reaction was terminated by the addition of 0.625 ml of 40% trichloroacetic acid and 0.3 ml 5 N hydrochloric acid. Vials were vortexed for 10 sec and 0.625 ml 2% thiobarbituric acid was added and mixed again. The mixture was then heated for 20 min at 95–100°C. The tubes were cooled and centrifuged for 10 min at 600 g and the absorbance of the supernate was measured at 585 nm. Malondialdehyde standards were prepared from 1,1,3,3-tetramethoxypropane and reacted similarly to obtain a calibration curve, which was used to calculate the amount of TBA reactive substances produced by silica samples.

#### *DNA strand breakage assay*

The DNA strand breakage assay was carried out according to methods described earlier [33, 34]. Briefly, reactions were carried out in 10 mM phosphate buffer (pH 7.4) in 1.5 ml polypropylene tubes at 37°C. Each reaction mixture contained 10  $\mu$ g DNA ( $\lambda$  Hind III digest) in a total volume of 100  $\mu$ l buffer. To this solution, 2  $\mu$ l of gel loading buffer (50 mM EDTA, 2.5% sodium dodecyl sulfate (SDS), 0.1% bromophenol) was added, and the sample then was electrophoresed in 0.7% agarose at 1–2 V/cm in 40 mM Tris-acetate buffer containing 2 mM EDTA (pH 8.0). Gels were stained in ethidium bromide (5  $\mu$ g/ml) for 10 minutes and photographed under ultraviolet transillumination.

#### *Cell line and cell culture*

Mouse macrophage cell line RAW 264.7 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were maintained in DMEM (Mediatech, Washington, DC, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1000 units/ml penicillin-streptomycin. For stimulation assay,  $5 \times 10^6$  RAW 264.7 cells were seeded into 6-well culture plates in 5 ml medium and stimulated with 100  $\mu$ g/ml silica or indicated agents for 6 h.

#### *Nuclear extracts*

Nuclear extracts were prepared by a modified method of Sun *et al.* [35]. RAW 264.7 cells were cultured in 6-well plates at  $2 \times 10^6$  cells/ml for 3 days, then the medium was replaced with fresh medium and cultured with 100  $\mu$ g/ml of silica combined with or without other agents as indicated for 6 h. At the end of the culture period, the cells were harvested and resuspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 M EDTA, 1 mM DTT, 0.5 mM PMSF) for 10 min on ice,

then vortexed for 10 sec. Nuclei were pelleted by centrifugation at  $12,000 \times g$  for 20 sec and were resuspended in buffer C (20 mM HEPES, pH 7.6, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for 30 min on ice. The supernates containing nuclear proteins were collected by centrifugation at  $12,000 \times g$  for 2 min and stored at  $-70^\circ\text{C}$ .

#### *Electrophoretic mobility shift assay (EMSA)*

EMSA is the method commonly used to measure NF- $\kappa$ B activation. The preparation of  $^{32}\text{P}$ -labeled double-stranded oligonucleotide containing NF- $\kappa$ B consensus sequence was performed as previously described [12]. Briefly, single-stranded DNA was synthesized based on the  $\kappa$ B-site in NF- $\kappa$ B2 (p100) gene promoter using a Millipore Cyclone Plus automated synthesizer. To prepare double-stranded DNA, the first strand DNA was annealed with a complementary decameric primer to its 3'-tail in  $2 \times$  anneal buffer. The second strand was extended with DNA polymerase Klenow fragment in a reaction mixture containing 250  $\mu$ Ci [ $^{32}\text{P}$ ]dCTP and 5 mM dATP, dGTP and dTTP. For EMSA, 4  $\mu$ g of nuclear extract was mixed with the labeled double-stranded probe and incubated at room temperature for 30 min. The reaction solution was electrophoresed on a native 6% polyacrylamide gel in  $0.25 \times$  TBE buffer for 2–3 h.

#### *TNF- $\alpha$ assay*

Mouse macrophage cell line RAW 264.7 cells were cultured at  $5 \times 10^6$ /ml in DMEM medium supported with 10% FBS in a 6-well tissue culture plate. After 72 h of incubation, the cells were stimulated with 20  $\mu$ g/ml silica in the absence or presence of various concentrations of aspirin for additional 6–12 h as indicated. TNF- $\alpha$  released to the media by macrophages were determined by immunological assay using TNF- $\alpha$  ELISA kit according to the manufacture's instruction (ENDOGEN, Cambridge, MA, USA).

The concentrations provided in Figure legends are final concentrations. All the experiments were carried out in air at room temperature except those specifically indicated.

## Results

#### *Scavenging of $\cdot\text{OH}$*

The Fenton reaction ( $\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \cdot\text{OH} + \cdot\text{OH}$ ) was used as a source of  $\cdot\text{OH}$  radicals. As shown in Figure 1a, an aqueous solution containing Fe(II),  $\text{H}_2\text{O}_2$ , and a spin trap (DMPO) in a phosphate buffer solution (pH 7.4), generated a 1:2:2:1 quartet with hyperfine splittings

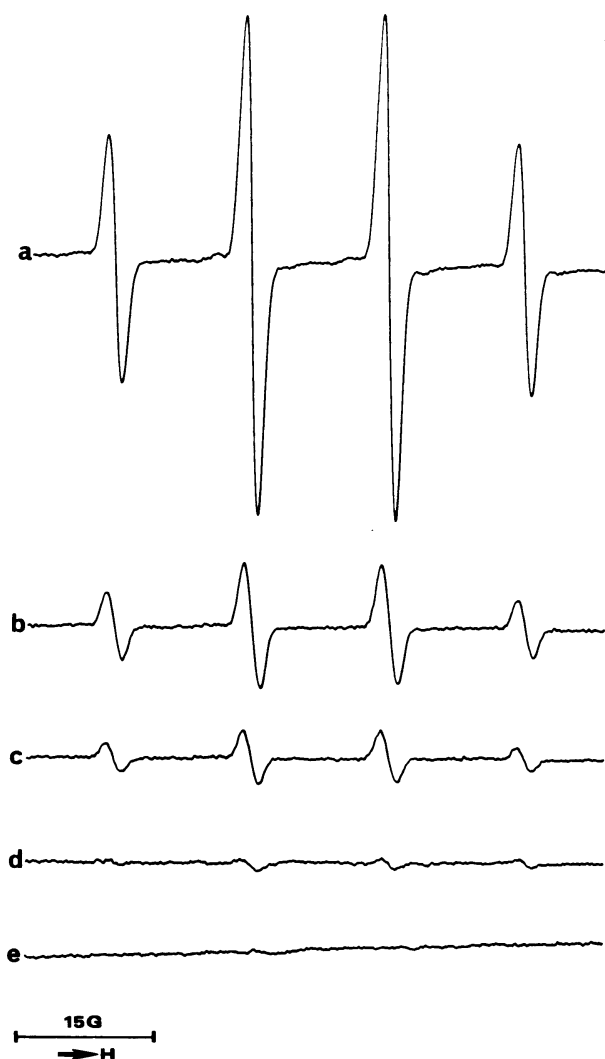
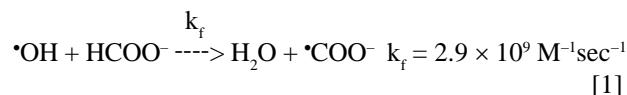


Fig. 1. ESR signals generated by DMPO/•OH adducts obtained from the reaction of Fe(II) with H<sub>2</sub>O<sub>2</sub>. (a) ESR spectrum recorded 1 min after mixing 1.0 mM FeCl<sub>2</sub>, 1.0 mM H<sub>2</sub>O<sub>2</sub>, and 1.0 mM DMPO in a pH 7.4 phosphate buffer solution. (b) Same as (a) but with 0.2 mM aspirin added. (c) Same as (a) but with 0.5 mM aspirin added. (d) Same as (a) but with 1.5 mM aspirin added. (e) Same as (a) but with 2.5 mM aspirin added. The spectrometer settings were: receiver gain,  $2.5 \times 10^4$ ; modulation amplitude, 0.5 G; magnetic field,  $3500 \pm 100$  G; scan time, 4 min.

of  $a_H = a_N = 14.9$  G, where  $a_H$  and  $a_N$  denote hyperfine splittings of  $\beta$ -hydrogen and nitroxyl nitrogen, respectively. Based on these splitting constants, this quartet spectrum was assigned to DMPO/•OH adduct, which is evidence for •OH generation [36]. As is noted from Figure 1b, 0.2 mM aspirin effectively reduced the intensity of the DMPO/•OH adduct. Increasing the aspirin concentration further decreased the intensity of the DMPO/•OH adduct (Figure 1c–d).

Spin trapping competition reactions were carried out using formate as another •OH radical scavenger to verify that the

decrease of the DMPO/•OH adduct is due to the •OH radical scavenging effect of aspirin and not due to the inhibition of •OH generation by aspirin. It is known that reaction of formate with •OH generates formate-derived (carbon-centered) radicals with a reaction rate of  $k_f = 2.9 \times 10^9 \text{ M}^{-1}\text{sec}^{-1}$  (Equation 1) [37, 38].



The newly generated •COO<sup>−</sup> radicals will react with DMPO to produce a DMPO/•COO<sup>−</sup> adduct. The reaction rate of DMPO with •OH is  $k_d = 2.1 \times 10^9 \text{ M}^{-1}\text{sec}^{-1}$  (Equation 2) [37, 38].



Because  $k_f$  and  $k_d$  are comparable, with excess amount of formate the •OH will predominately react with formate to



Fig. 2. Effect of aspirin on the DMPO/•COO<sup>−</sup> adduct. (a) ESR spectrum recorded 1 min after mixing 1.0 mM DMPO, 1.0 mM FeCl<sub>2</sub>, 1.0 mM H<sub>2</sub>O<sub>2</sub>, and 50 mM sodium formate in a pH 7.4 phosphate-buffered solution. (b) Same as (a) but with 1.0 mM aspirin added. The spectrometer settings were: receiver gain,  $2.5 \times 10^4$ ; modulation amplitude, 0.5 G; magnetic field,  $3500 \pm 100$  G; scan time, 4 min.

generate  $\bullet\text{COO}^-$ . In this case, the spin adducts obtained should mostly be  $\text{DMPO}/\bullet\text{COO}^-$ . As shown in Fig. 2a, reaction of  $\text{Fe(II)}$  with  $\text{H}_2\text{O}_2$  in the presence of DMPO and 50 mM formate generates  $\text{DMPO}/\bullet\text{COO}^-$  as a major spin adduct signal. If aspirin blocked the  $\bullet\text{OH}$  generation, the  $\text{DMPO}/\bullet\text{COO}^-$  signal should not be observed. As shown in Fig. 2b, addition of aspirin only slightly decreased the intensity of  $\text{DMPO}/\bullet\text{COO}^-$ . Thus, the results show that aspirin is capable of scavenging the  $\bullet\text{OH}$  radicals and does not block their generation. These results also show that aspirin does not significantly react with  $\text{H}_2\text{O}_2$ . Otherwise, the  $\bullet\text{OH}$  radical generation would be inhibited.

The change in fluorescence of scopoletin in the presence of horseradish peroxidase was used to further evaluate whether aspirin is able to react with  $\text{H}_2\text{O}_2$ . At a concentration of 2.0 mM aspirin only decreased the concentration of  $\text{H}_2\text{O}_2$  from 0.758–0.743 mM, a change of about 2%, showing that aspirin is not a good scavenger for  $\text{H}_2\text{O}_2$ .

We have also investigated the superoxide ( $\text{O}_2^-$ ) scavenging activity of aspirin using xanthine (0.2 mM) and xanthine oxidase (0.1 U/ml) as a source of  $\text{O}_2^-$  radicals. A mixture of xanthine and xanthine oxidase in the presence of DMPO generated a typical  $\text{DMPO}/\text{O}_2^-$  spin adduct signal (data not shown). Addition of 2.0 mM aspirin to this mixture caused less than 4% reduction in the  $\text{DMPO}/\text{O}_2^-$  signal intensity, indicate that aspirin is not an efficient  $\text{O}_2^-$  radical scavenger.

#### Calculation of reaction rate constant of aspirin with $\bullet\text{OH}$ radical

Kinetic studies according to methods reported earlier for the reaction of ethanol with  $\bullet\text{OH}$  were carried to determine the reaction rate of aspirin with  $\bullet\text{OH}$  radicals [38, 39]. The reaction steps may be written as:



$$-d[\bullet\text{OH}]/dt = k_d[\text{DMPO}][\bullet\text{OH}] + k_a[\bullet\text{OH}][\text{aspirin}] \quad [4]$$

$$d[\text{DMPO}/\bullet\text{OH}]/dt = k_d[\text{DMPO}][\bullet\text{OH}] \quad [5]$$

Dividing equation [4] by equation [5], one obtains equation [6].

$$\frac{-d[\bullet\text{OH}]/dt}{d[\text{DMPO}/\bullet\text{OH}]/dt} = 1 + \frac{k_a[\text{aspirin}]}{k_d[\text{DMPO}]} \quad [6]$$

At a saturating level of DMPO and in the absence of aspirin, the rate of  $\bullet\text{OH}$  spin trapping is equal to the rate of  $\bullet\text{OH}$  generation,  $d[\bullet\text{OH}]/dt$ . If  $V$  and  $v$  represent the rate of  $\bullet\text{OH}$

spin trapping in the absence and in the presence of aspirin, respectively, one obtains equation [7].

$$V/v = 1 + k_a[\text{aspirin}]/k_d[\text{DMPO}] \quad [7]$$

or

$$V/v - 1 = \frac{k_a[\text{aspirin}]}{k_d[\text{DMPO}]} \quad [8]$$

Figure 3 shows the scavenging of  $\bullet\text{OH}$  by aspirin. The data were plotted according to equation [8]. A straight line is obtained with a slope of 17.0, which is the ratio of  $k_a/k_d$  (Fig. 3). Using the value of  $k_d = 2.1 \times 10^9 \text{ M}^{-1}\text{sec}^{-1}$  for the  $\bullet\text{OH}$  trapping by DMPO [37], the value of  $k_a$  is calculated as follows:

$$\begin{aligned} k_a &= 17 k_d \\ &= 17 \times 2.1 \times 10^9 \text{ M}^{-1}\text{sec}^{-1} \\ &= 3.57 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1} \end{aligned}$$

It should be noted that the rate constant  $k_a$  as calculated above may not be very accurate. It could be in the range of  $3.00 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$  –  $5.00 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ . For example, this method did not include the decay of the spin adduct after its formation [40]. However, it does provide a convenient method for obtaining a relative value.

#### Inhibition of silica-induced lipid peroxidation

Our earlier studies [41] have shown that silica is able to induce lipid peroxidation via  $\bullet\text{OH}$  radical-initiated free radical

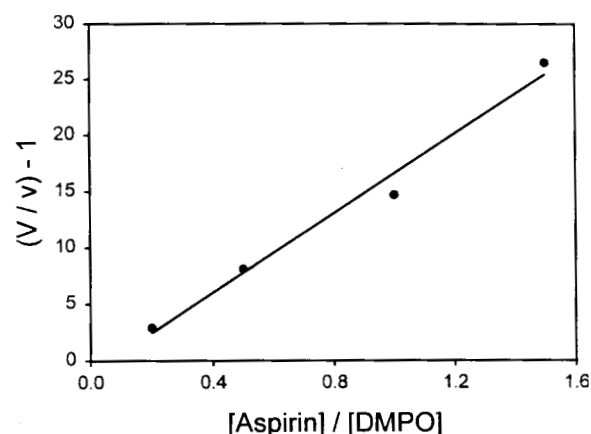


Fig. 3. Scavenging of  $\bullet\text{OH}$  radicals by aspirin. The  $\bullet\text{OH}$  radicals were produced by the reaction of 1.0 mM  $\text{FeCl}_2$  with 1.0 mM  $\text{H}_2\text{O}_2$  in the presence of 1.0 mM DMPO. The data were plotted according to  $V/v - 1 = k_a[\text{aspirin}]/k_d[\text{DMPO}]$ , as explained in the text.

reactions. In the present studies, we evaluated the effect of aspirin on silica-induced lipid peroxidation. As shown in Fig. 4, silica particles caused lipid peroxidation by formation of thiobarbituric acid substances. Aspirin exhibited a dose-dependent inhibition of silica-induced lipid peroxidation (Fig. 4).

#### *Inhibition of silica-induced DNA strand breaks*

As shown in Fig. 5, lanes 1 and 2, incubation of DNA or DNA with  $H_2O_2$  for 24 h did not exhibit any observable DNA strand breaks. However, silica plus  $H_2O_2$  caused a significant degree of DNA strand breaks (Fig. 5, lane 3). Catalase blocked induction of strand breaks (Fig. 5, lane 4). Aspirin partially inhibited the DNA strand breaks with 0.2 mM aspirin exhibiting a noticeable inhibitory effect (Fig. 5, lanes 5 and 6).

#### *Inhibition of NF- $\kappa$ B activation*

The mouse macrophage cell line RAW 264.7 cells were used to detect NF- $\kappa$ B activation by silica, LPS and Fe(II). The cells were exposed for 6 h, and NF- $\kappa$ B was analyzed in the nuclear extracts. As shown in Fig. 6a, lane 1, untreated cells did not exhibit any significant activation of NF- $\kappa$ B activity (relatively density photometer reading,  $1.0 \pm 0.1$ ). Upon treatment with silica, the cells enhanced NF- $\kappa$ B binding activity (Fig. 6a, lane 2) (relatively density photometer reading,  $5.0 \pm 0.4$ ). Aspirin partially inhibited this

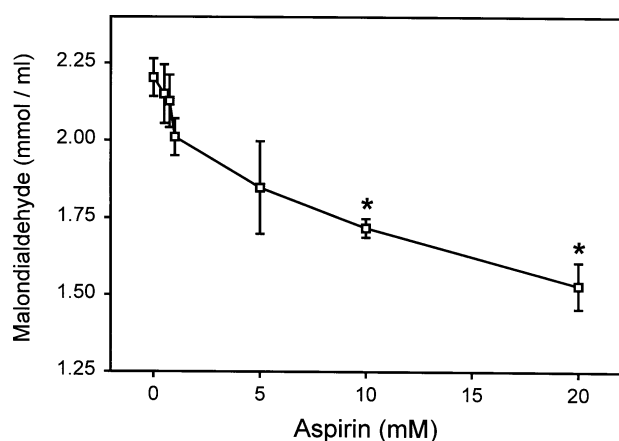


Fig. 4. Silica-induced lipid peroxidation and its inhibition by aspirin. Incubation mixture contained 10 mg/ml silica particles, 100  $\mu$ l of 0.9 M linoleic acid, HEPES-buffered medium and different concentrations of aspirin as indicated in a total volume of 1 ml. Data presented are the means of  $\pm$  S.D. of a minimum of five sets of experiments performed in duplicate. Other experimental conditions are described in the section of Materials and methods. Asterisks indicate a significant decrease in lipid peroxidation from control ( $p < 0.05$ ).

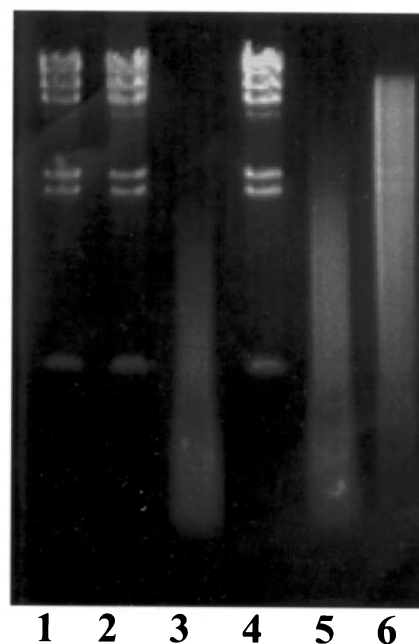


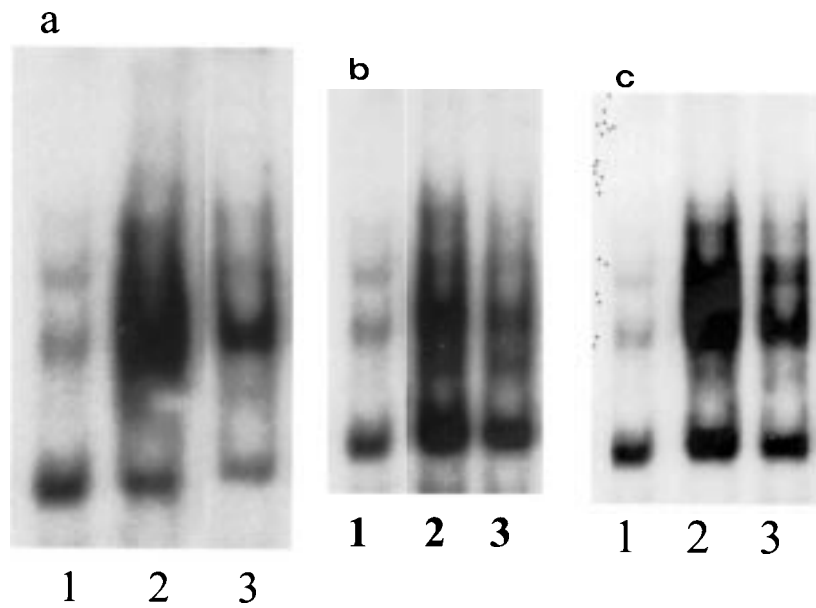
Fig. 5. DNA strand breaks induced by silica. Lane 1, untreated  $\lambda$  Hind III digested DNA in a pH 7.4 phosphate buffer solution; lane 2, DNA + 1%  $H_2O_2$ ; lane 3, 20  $\mu$ g/ml silica + 1%  $H_2O_2$ ; lane 4, 20  $\mu$ g/ml silica + 1%  $H_2O_2$  + 5,000 units/ml catalase; lane 5, 20  $\mu$ g/ml silica + 1%  $H_2O_2$  + 0.02 mM aspirin; lane 6, 20  $\mu$ g/ml silica + 1%  $H_2O_2$  + 0.2 mM aspirin. All the samples were incubated for 24 h at 37°C. Other experimental conditions were described in the Materials and methods.

silica-induced NF- $\kappa$ B activation (Fig. 6a, lane 3) (relatively density photometer reading,  $1.6 \pm 0.2$ ).

LPS and Fe(II) were also used as NF- $\kappa$ B stimuli. These agents are known to cause NF- $\kappa$ B activation which can be inhibited by antioxidants. As shown in Fig. 6b, LPS is indeed able to induce NF- $\kappa$ B activation (relatively density photometer reading,  $3.7 \pm 0.3$ ) and aspirin suppressed this activation (relatively density photometer reading,  $1.7 \pm 0.2$ ). Similarly, aspirin also inhibited Fe(II) induced NF- $\kappa$ B activation (Fig. 6b and c) (relatively density photometer readings,  $3.8 \pm 0.3$  via  $1.6 \pm 0.2$ ).

#### *Inhibition of silica-induced TNF- $\alpha$ production*

Although cells alone incubated for 6 h produced TNF- $\alpha$ , silica particles enhanced its production (Fig. 7a). Aspirin caused a dose-dependent inhibition on silica-induced TNF- $\alpha$  production. Similar results were obtained for 12 h of incubation (Fig. 7b).

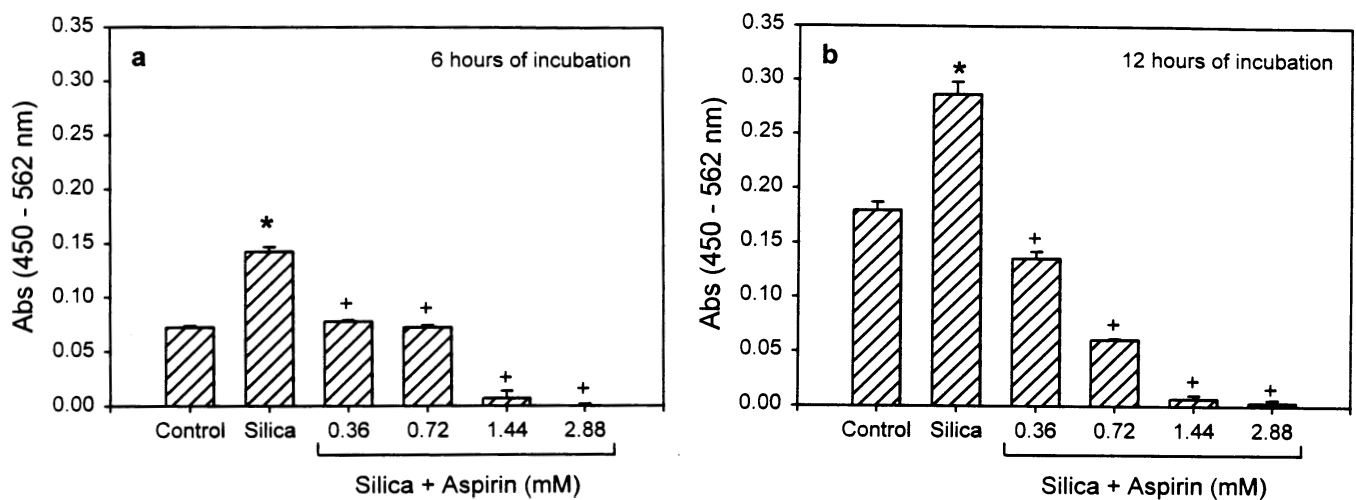


**Fig. 6.** Effect of aspirin on DNA binding activity of NF-κB protein induced by silica, LPS and Fe(II). The RAW 264.7 cells were adjusted to a density of  $5 \times 10^6/\text{ml}$  and treated for 6 h with different stimuli, then subjected to extraction of nuclear proteins as described in the Materials and methods. The DNA binding activity of NF-κB protein was detected with a probe of  $^{32}\text{P}$ -labeled double stranded NF-κB binding oligonucleotide by electrophoretic mobility shift assay. (a) Lane 1, untreated cells; lane 2, cells + 20  $\mu\text{g}/\text{ml}$  silica; lane 3, cells + 20  $\mu\text{g}/\text{ml}$  silica + 4 mM aspirin. (b) Lane 1, untreated cells; lane 2, cells + 5  $\mu\text{g}/\text{ml}$  LPS; lane 3, cells + 5  $\mu\text{g}/\text{ml}$  LPS + 4 mM aspirin. (c) Lane 1, untreated cells; lane 2, cells + 0.1 mM Fe(II); lane 3, cells + 0.1 mM Fe(II) + 2 mM aspirin.

## Discussion

Using ESR spin trapping, the present study shows that aspirin is an efficient  $\cdot\text{OH}$  radical scavenger. The reaction rate constant

is  $3.6 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ . The  $\cdot\text{OH}$  scavenging activity of aspirin is better than several well established antioxidants, such as ascorbate ( $1.2 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ ), GSH ( $1.5 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ ) and cysteine ( $1.3 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ ) (Table 1). An antioxidant is



**Fig. 7.** Effect of aspirin on silica-induced TNF-α production. The RAW 264.7 cells were cultured at  $5 \times 10^6/\text{ml}$  DMEM medium supported with 10% FCS in a 6-well tissue culture plate. After 72 h of incubation, the cells were stimulated with 20  $\mu\text{g}/\text{ml}$  silica in the absence and presence of various concentrations of aspirin for an additional 6 (a) and 12 h (b) as indicated. TNF-α released to the medium by macrophages were determined by using a TNF-α ELISA kit. Values are means and standard deviation of three separate experiments. Asterisks indicate a significant increase from control, while plus signs indicate a significant decrease from silica alone ( $p < 0.05$ ).

Table 1. Reaction rate constants of some antioxidants with  $\bullet\text{OH}$  radical.

| Antioxidant | k ( $\text{M}^{-1}\text{sec}^{-1}$ ) | Reference |
|-------------|--------------------------------------|-----------|
| ascorbate   | $1.3 \times 10^{10}$                 | 42        |
| glutathione | $1.5 \times 10^{10}$                 | 42        |
| cysteine    | $1.5 \times 10^{10}$                 | 42        |
| azide       | $1.1 \times 10^{10}$                 | 42        |
| lipoic acid | $1.9 \times 10^{10}$                 | 43        |
| aspirin     | $3.6 \times 10^{10}$                 | This work |

defined as any chemical or biological species which reacts efficiently with an oxidant, especially reactive oxygen species, to protect an oxidizable target from damage by this oxidant. It appears that aspirin may inhibit oxidant-induced cellular damage predominantly via scavenging  $\bullet\text{OH}$  radicals and not via attenuating its generation as supported by competition reactions using excess formate.

The antioxidant property of aspirin may explain many of its biological properties. It has been reported that aspirin is able to inhibit NF- $\kappa$ B activation caused by phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) [44]. This transcription factor is critical for the inducible expression of multiple cellular and viral genes involved in inflammation and infection including interleukin-I (IL-1), IL-6, TNF- $\alpha$ , and adhesion molecules. It is generally believed that antioxidants are potent inhibitors of NF- $\kappa$ B activation due to their ability to scavenge radicals generated in response to PMA or LPS [45]. In the present study, several NF- $\kappa$ B stimuli, silica, LPS and Fe(II) were used. All of these stimuli were able to cause NF- $\kappa$ B activation via oxidative stress. In a recent study, it has been reported that among reactive oxygen species,  $\bullet\text{OH}$  radical functions as a messenger for NF- $\kappa$ B activation [46]. The present study shows that aspirin did not react significantly with  $\text{H}_2\text{O}_2$  or  $\text{O}_2^-$ . While other mechanisms may be involved in the inhibition by aspirin, it seems likely that aspirin's  $\bullet\text{OH}$  radical scavenging property plays an important role. Similarly, aspirin is also able to inhibit activator protein (AP-1) and neoplastic transformation induced by a tumor promoter [47]. This transcription factor is also critical for induction of neoplastic transformation and induction of multiple genes involved in inflammation and infection. Free radicals are considered to be important messengers in AP-1 activation [16]. The free radical scavenging property of aspirin is also likely to be involved in its ability to inhibit AP-1 activation.

In the present study, we have chosen silica-induced lipid peroxidation and its inhibition by aspirin as an example to further demonstrate the antioxidant property of aspirin. The results show that silica particles react with linoleic acid to induce lipid peroxidation and that aspirin significantly inhibits this reaction. It may be noted that when quartz is fractured in air, siliconbased radicals are generated on the

surface of quartz [29, 41]. These radicals subsequently react with atmospheric oxygen and water to generate  $\text{H}_2\text{O}_2$ ,  $\bullet\text{OH}$ , and  $\text{O}_2^-$ . In addition, iron and other transition metals that may be present as trace impurities on the silica can surface catalyze the generation of  $\bullet\text{OH}$  radicals from  $\text{H}_2\text{O}_2$ . The free radicals ( $\text{Si}^\bullet$  and  $\text{SiO}^\bullet$ ) on the surface of silica particles and associated oxygenated reactive species could perturb the cell membrane and initiate lipid peroxidation [29]. The result would be the release of reactive oxygenated species ( $\text{H}_2\text{O}_2$ ,  $\text{O}_2^-$ ,  $\bullet\text{OH}$ , and  $\text{RO}^\bullet$ ). These reactive oxygenated species would further react with the cell membrane, leading to an additional release of these species. This process has been suggested as a primary step in the pathogenesis of silicosis [29]. The inhibition of silica-induced reactions by aspirin may attenuate or prevent silica-induced lipid peroxidation and protect the cell membrane from silica-induced oxidative damage.

As stated in the Introduction, silica is a fibrogenic agent due to its ability to elicit resident macrophages to release inflammatory mediators and cytokines which can promote fibroblast proliferation and collagen deposition. It has been suggested that NF- $\kappa$ B activation is crucial to cytoplasmic/nuclear signaling in cells exposed to injury-producing conditions [48]. NF- $\kappa$ B serves as a second messenger to induce a series of cellular genes in response to an environmental perturbation. Among cellular genes regulated by NF- $\kappa$ B are several proinflammatory and cytotoxic cytokines, including IL-2, IL-6 and TNF- $\alpha$  (49). NF- $\kappa$ B activates these genes by acting as a transcriptional factor and binding to the NF- $\kappa$ B consensus sequence in their promoters. Inhibition of NF- $\kappa$ B activation may inhibit TNF- $\alpha$  production. As shown in the present study, aspirin effectively blocked silica-induced TNF- $\alpha$  production. Although further study is required, it is likely that the inhibition of TNF- $\alpha$  production by aspirin involves NF- $\kappa$ B inhibition. Since silica-induced DNA damage, NF- $\kappa$ B activation and TNF- $\alpha$  activation are involved in the mechanism of silica-induced fibrogenicity and carcinogenicity, aspirin may be a potential therapeutical agent against silica-induced cellular injury.

In conclusion, the results obtained from the present study show that aspirin is an efficient  $\bullet\text{OH}$  radical scavenger with reaction rate constant of  $k = 3.6 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ , which is faster than several well established antioxidants, such as ascorbate, glutathione and cysteine. Aspirin inhibited silica-induced lipid peroxidation and DNA strand breaks. Aspirin also inhibited the activation of NF- $\kappa$ B induced by silica, LPS and Fe(II), and the production of TNF- $\alpha$  induced by silica. The results indicate that the antioxidant property of aspirin may explain in part its various pharmacological actions.



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