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Inhibition of peroxynitrite-mediated DNA strand cleavage and hydroxyl radical formation by aspirin at pharmacologically relevant concentrations: Implications for cancer intervention

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ABSTRACT

Epidemiological studies have suggested that the long-term use of aspirin is associated with a decreased incidence of human malignancies, especially colorectal cancer. Since accumulating evidence indicates that peroxynitrite is critically involved in multistage carcinogenesis, this study was undertaken to investigate the ability of aspirin to inhibit peroxynitrite-mediated DNA damage. Peroxynitrite and its generator 3-morpholinosydnonimine (SIN-1) were used to cause DNA strand breaks in φX-174 plasmid DNA. We demonstrated that the presence of aspirin at concentrations (0.25–2 mM) compatible with amounts in plasma during chronic anti-inflammatory therapy resulted in a significant inhibition of DNA cleavage induced by both peroxynitrite and SIN-1. Moreover, the consumption of oxygen caused by 250 μM SIN-1 was found to be decreased in the presence of aspirin, indicating that aspirin might affect the auto-oxidation of SIN-1. Furthermore, EPR spectroscopy using 5,5-dimethylpyrroline-*N*-oxide (DMPO) as a spin trap demonstrated the formation of DMPO-hydroxyl radical adduct (DMPO-OH) from authentic peroxynitrite, and that aspirin at 0.25–2 mM potently diminished the radical adduct formation in a concentration-dependent manner. Taken together, these results demonstrate for the first time that aspirin at pharmacologically relevant concentrations can inhibit peroxynitrite-mediated DNA strand breakage and hydroxyl radical formation. These results may have implications for cancer intervention by aspirin.

Introduction

Acetylsalicylic acid (aspirin) is a commonly used non-steroidal, analgesic and anti-inflammatory drug with a wide spectrum of pharmacological effects [1,2]. Recent studies have indicated that the long-term use of aspirin in humans can reduce the risk of colorectal cancer as well as malignancies in other tissues [3,4]. In addition, aspirin has been demonstrated to inhibit chemically induced carcinogenesis in various animal models [5,6]. The protective effects of aspirin against carcinogenesis are believed to result from its ability to inhibit inflammation [7]. The anti-inflammatory action of aspirin are attributed to its ability to irreversibly inhibit cyclooxygenase activity and reduce the synthesis of pro-inflammatory prostaglandins [7,8]. Moreover, some previous studies reported that aspirin can inhibit the activation of NF-κB, a transcription factor critically involved in the production of inflammatory cytokines, and the subsequent inflammatory responses [9,10].

Cumulative evidence has indicated that oxidative stress plays important roles in the progression of many chronic diseases including cancer, cardiovascular diseases, neurodegenerative disorders and diabetes [11–14]. One such mediator of oxidative stress is peroxynitrite ($ONOO^-$), which may be generated by activated macrophages releasing both nitric oxide and superoxide [15,16]. Peroxynitrite can impair most cellular components, including DNA, proteins, and phospholipid membranes [17,18]. The mechanisms account for cytotoxicity elicited by peroxynitrite are multiple, among them, induction of DNA strand breaks and the subsequent activation of poly (ADP-ribose) polymerase have been demonstrated to be critical events [19]. In view of the critical involvement of oxidative stress in multistage carcinogenesis, we hypothesized that aspirin may protect against carcinogenesis by inhibiting oxidative DNA damage induced by peroxynitrite. To test this hypothesis, in this study φX-174 plasmid DNA was used as a

Abbreviations: DMPO, 5,5-dimethylpyrroline-*N*-oxide; DMPO-OH, DMPO-hydroxyl adduct; DMPO-OOH, DMPO-superoxide spin adduct; EPR, electron paramagnetic resonance; SOD, superoxide dismutase; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine.

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model system; peroxynitrite and its generator 3-morpholinosydnonimine (SIN-1) were used to elicit DNA damage. We observed that aspirin at pharmacologically relevant concentrations significantly inhibited the oxidative DNA cleavage and hydroxyl radical formation mediated by peroxynitrite.

Materials and methods

Reagents. Peroxynitrite was from Calbiochem (San Diego, CA). φX-174 RF I DNA and Lambda DNA–HindIII digest were obtained from New England Biolabs (Beverley, MA). Aspirin, SIN-1 and other chemicals were purchased from Sigma (St. Louis MO).

Preparation of SIN-1 and peroxynitrite. SIN-1 was dissolved in phosphate–buffer saline (PBS, pH 5.5), and stored at −80 °C. The concentration of peroxynitrite was measured at 302 nm and calculated using a molar extinction coefficient of 1670 M^{-1} cm^{-1}. Aliquots of peroxynitrite were stored at −80 °C.

DNA cleavage assay. DNA cleavage was assessed by detecting the conversion of supercoiled φX-174 RF I DNA double-stranded DNA to open circular and linear forms [20]. Briefly, 0.2 μg DNA was incubated with SIN-1 or peroxynitrite in the presence or absence of aspirin in PBS (pH 7.4) at 37 °C (for SIN-1) or room temperature (for peroxynitrite). To prevent the change of pH of the reaction mixture, the stock solution of aspirin was adjusted to pH 7.4 with NaOH. Following incubation, the samples were immediately loaded into 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA and electrophoresed in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer. After electrophoresis, the gels were stained with 0.5 μg ml^{-1} ethidium bromide for 30 min, followed by another 30 min destaining in water. The gels were then photographed under ultraviolet illumination and quantified using Alpha Innotech Imaging system (San Leandro, CA).

Measurement of oxygen consumption. Oxygen consumption caused by SIN-1 auto-oxidation was monitored with a Clark oxygen electrode (YSI 5300, Yellow Springs, OH) as described previously [20]. Briefly, aspirin was dissolved in distilled water. The reaction mixtures containing 2.5 ml PBS, pH 7.4 were added into the Perspex incubation chamber at 37 °C in the presence or absence of various concentrations of aspirin. The Perspex incubation chamber was then closed by the Clark electrode that does not allow ambient oxygen to access the measurement volume. Therefore, the decay of oxygen within the chamber directly relates to the actual oxygen consumption. After stabilization of the reaction mixture for 5 min, reactions were started by the addition of SIN-1. The oxygen consumption was expressed as percentage of saturation oxygen.

Electron paramagnetic resonance (EPR) spin-trapping assay. For 5,5-dimethylpyrroline-*N*-oxide (DMPO)-spin trapping measurement of hydroxyl radicals, EPR spectra were recorded at room temperature using a spectrometer (Bruker D-200 ER, IBMBruker), operating at X-band with a TM cavity and capillary tube, as described previously [21,22]. For peroxynitrite reaction, the EPR spectrometer settings were: modulation frequency, 100 kHz; X-band microwave frequency, 9.5 GHz; microwave power, 15 mW (milliwatts); modulation amplitude, 6.3 G (gauss); time constant, 160 s; scan time, 200 s; and receiver gain, $4 \times l0^5$. For the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$), the EPR spectrometer settings were: modulation frequency, 100 kHz; X-band microwave frequency, 9.5 GHz; microwave power, 15 mW (milliwatts); modulation amplitude, 1.0 G (gauss); time constant, 160 s; scan time, 200 s; and receiver gain, $1 \times l0^5$. Reactants were mixed in test tubes in a final volume of 100 μl and the reaction mixture was then transferred to a capillary tube for EPR detection at room temperature under conditions described above. Spectral simulations were performed on the EPR data by matching directly to the spectra as described previously [23].

Statistical analysis. Unless otherwise stated, data were expressed as means ± SD and analyzed statistically by one-way ANOVA. $p < 0.05$ was considered statistically significant.

Results and discussion

DNA cleavage elicited by SIN-1

Induction of single-strand breaks to the supercoiled double-stranded φX-174 RF I DNA leads to generation of open circular DNA, while the generation of a linear form of DNA is indicative of double-strand breaks [24]. At a physiological pH, SIN-1 is believed to undergo auto-oxidation to produce equal nitric oxide and superoxide, leading to the formation of peroxynitrite [25,26]. Because the formation of peroxynitrite from SIN-1 auto-oxidation mimics the in vivo formation of peroxynitrite, SIN-1 is commonly used as a peroxynitrite generator for studying peroxynitrite-mediated biological effects [25,26]. As shown in Fig. 1A, incubation of the plasmid DNA with 0–1000 μM SIN-1 resulted in a significant formation of both open circular and linear forms of DNA in a concentration- and time-dependent manner. These results indicated that SIN-1 was able to elicit both single- and double-stranded breaks in this DNA system. As significant amounts of both open circular and linear forms of DNA were observed following incubation of φX-174 RF I DNA with 250 μM SIN-1 for 60 min (Fig. 1A(b)), the above concentration of SIN-1 and incubation time were selected in subsequent experiments to determine the protective effects of aspirin.

SIN-1-mediated DNA cleavage in the presence of aspirin

We observed that SIN-1-mediated single- as well as double-stranded DNA breaks were significantly inhibited by aspirin in a concentration-dependent manner. A remarkable inhibition of SIN-1-induced DNA cleavage was detected with aspirin at 0.5 mM. Further decreases of both single- and double-stranded DNA cleavage were observed when the concentrations of aspirin increased from 0.5 to 2 mM (Fig. 1B).

SIN-1-mediated oxygen consumption in the presence of aspirin

Oxygen utilization is a critical event leading to the formation of peroxynitrite during SIN-1 auto-oxidation [27]. Accordingly, we examined the effect of aspirin on SIN-1-mediated oxygen utilization. Addition of SIN-1 (0–1000 μM) to PBS (pH 7.4) resulted in oxygen utilization in a concentration-dependent fashion. The oxygen consumption caused by 250 μM SIN-1 was decreased in the presence of aspirin (0.25–2 mM) (Fig. 1C), suggesting that aspirin at the above concentrations might affect the auto-oxidation of SIN-1. While the mechanism by which aspirin inhibits SIN-1-mediated oxygen consumption remains unclear, the inhibition of SIN-1-mediated oxygen consumption by aspirin indicated that the protective effects of aspirin (0.25–2 mM) on SIN-1-induced DNA strand breakage might result from the diminished auto-oxidation of SIN-1 in the presence of aspirin. Therefore, we next determined whether aspirin also inhibited the DNA strand breaks induced by authentic peroxynitrite.

Peroxynitrite-mediated DNA strand breaks in the presence of aspirin

To further demonstrate the protective effect of aspirin on peroxynitrite-induced DNA strand breaks, authentic peroxynitrite was used to trigger DNA strand breaks [21]. As shown in Fig. 2A and B, incubation of the plasmid DNA with peroxynitrite at room temperature for 60 min resulted in an increased formation of open cir-

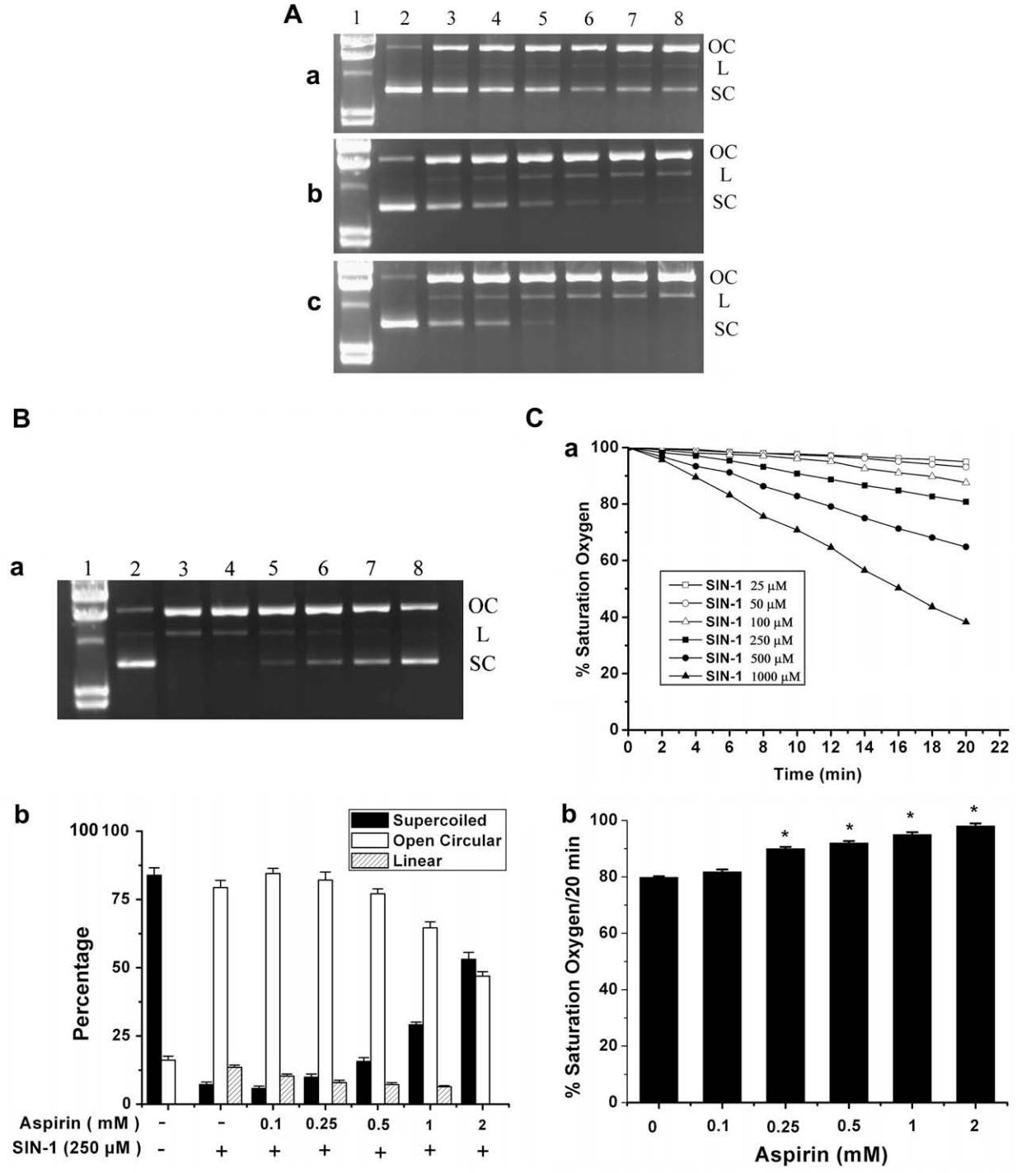

Fig. 1. DNA damage induced by SIN-1 in the presence or absence of aspirin. (A) Concentration- and time-dependent induction of DNA strand breaks by SIN-1. φX-174 RF I plasmid DNA was incubated with indicated concentrations of SIN-1 for 30 min (a), 60 min (b), and 120 min (c). lane 1: marker; lane 2: control; lane 3: SIN-1 25 μM; lane 4: SIN-1 50 μM; lane 5: SIN-1 100 μM; lane 6: SIN-1 250 μM; lane 7: SIN-1 500 μM; lane 8: SIN-1 1000 μM. The marker used was Lambda DNA–HindIII digest. OC, L, and SC stand for open circular, linear, and supercoiled DNA, respectively. (B) Effects of aspirin on SIN-1-induced DNA damage. φX-174 RF I plasmid DNA was incubated with SIN-1 in the presence or absence of the indicated concentrations of aspirin for 60 min. (a) A representative agarose gel. lane 1: marker; lane 2: control; lane 3: SIN-1 250 μM; lane 4: SIN-1 250 μM + aspirin 0.1 mM; lane 5: SIN-1 250 μM + aspirin 0.25 mM; lane 6: SIN-1 250 μM + aspirin 0.5 mM; lane 7: SIN-1 250 μM + aspirin 1 mM; lane 8: SIN-1 250 μM + aspirin 2 mM. The marker used was Lambda DNA–HindIII digest. OC, L, and SC stand for open circular, linear, and supercoiled DNA, respectively. (b) Quantitative analysis for the protection by different concentrations of aspirin against the DNA damage at 250 μM SIN-1 with 60 min exposure. (C) Effects of aspirin on SIN-1-mediated oxygen consumption. The oxygen consumption was measured following incubation of SIN-1 in the presence or absence of the indicated concentrations of aspirin for 20 min. (a) The representative oxygen consumption curves with indicated concentrations of SIN-1. (b) Effect of aspirin on the oxygen consumption caused by 250 μM SIN-1. All data represent similar results from three independent experiments. Data of columns represent means ± SD of three independent experiments ($^{*}p < 0.05$ versus control).

cular form of DNA in a concentration-dependent fashion. A significant increased formation of open circular form of DNA was observed with 100 μM peroxynitrite following 60 min incubation at room temperature. These results demonstrated that addition of peroxynitrite to plasmid DNA could induce single-stranded breaks. Similar results are also found in some previous studies [27,28]. Fur-

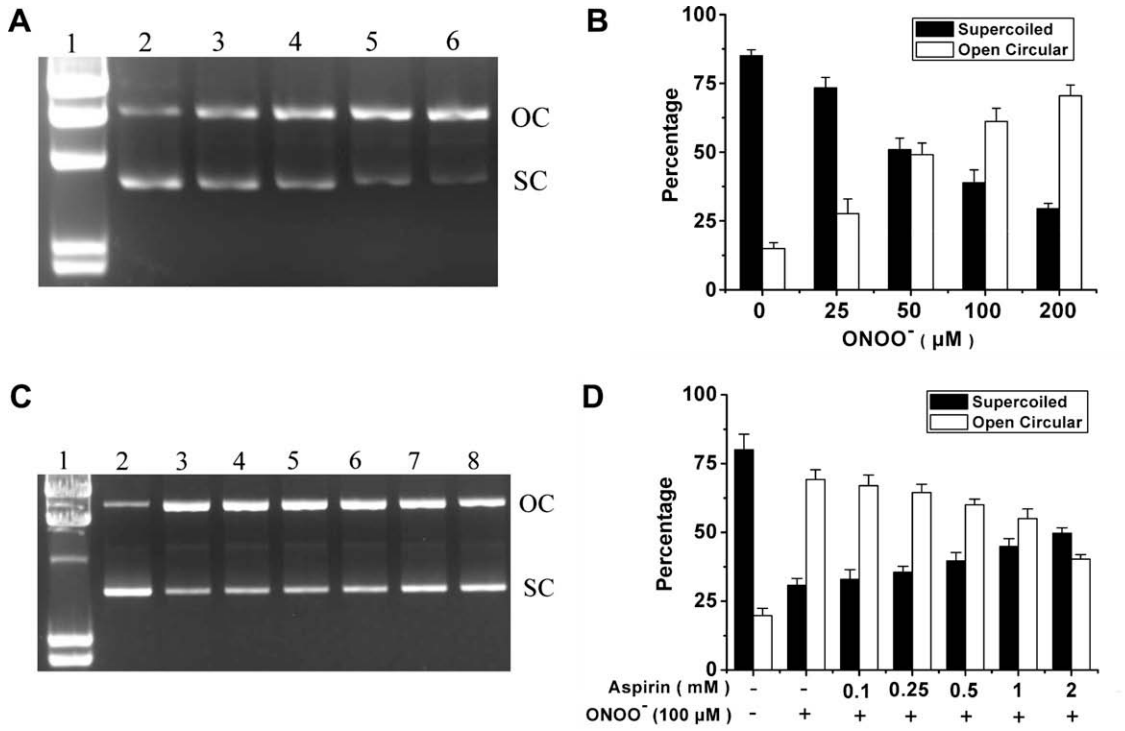

Fig. 2. DNA damage induced by peroxynitrite in the presence or absence of aspirin. φX-174 RF I plasmid DNA was incubated with peroxynitrite ($ONOO^-$) in the presence or absence of the indicated concentrations of aspirin for 60 min as described in the text. (A) A representative agarose gel picture of the φX-174 RF I plasmid DNA after incubation with the indicated concentrations of peroxynitrite for 60 min. lane 1: marker; lane 2: control; lane 3: $ONOO^-$ 25 μM; lane 4: $ONOO^-$ 50 μM; lane 5: $ONOO^-$ 100 μM; lane 6: $ONOO^-$ 200 μM. (B) The quantitative data of panel A. (C) A representative gel picture of the φX-174 RF I plasmid DNA after incubation with 100 μM peroxynitrite in the presence or absence of the indicated concentrations of aspirin for 60 min. lane 1: marker; lane 2: control; lane 3: $ONOO^-$ 100 μM; lane 4: $ONOO^-$ 100 μM + aspirin 0.1 mM; lane 5: $ONOO^-$ 100 μM + aspirin 0.25 mM; lane 6: $ONOO^-$ 100 μM + aspirin 0.5 mM; lane 7: $ONOO^-$ 100 μM + aspirin 1 mM; lane 8: $ONOO^-$ 100 μM + aspirin 2 mM. (D) The quantitative data of panel C. The data of both panel B and D represent means ± SD of three independent experiments.

ther study showed that the single-stranded DNA breaks caused by 100 μM peroxynitrite could be inhibited by aspirin in a concentration-dependent manner (Fig. 2C and D). Notably, a significant protection was observed with 0.5 mM aspirin, demonstrating that aspirin could be a potent inhibitor of peroxynitrite-mediated DNA damage.

Peroxynitrite-mediated formation of hydroxyl radicals in the presence of aspirin

Peroxynitrite decomposition has been suggested to generate free radicals which can cause DNA damage in multiple types of cells [16]. Therefore, EPR studies were employed to examine the effect of aspirin on peroxynitrite-mediated free radical formation using the spin trap DMPO, which reacts with oxygen radicals, including superoxide and hydroxyl radicals to generate relatively stable spin-adducts [29]. As shown in Fig. 3 (lines c and d), a well characterized 1:2:2:1 pattern of DMPO-hydroxyl adduct (DMPO-OH) with the hyperfine splitting constants ($a_N = a_H = 14.9$ gauss (G)) was observed after incubation with 100 and 1000 μM peroxynitrite, respectively. DMPO itself did not give rise to the formation of any detectable spin-adducts (Fig. 3 line a), indicating the high purity of this spin trap used in this study. Previous studies reported that DMPO-OH could also result from decomposition of the superoxide spin-adduct (DMPO-OOH) [30]. To examine whether DMPO-OH formation is through the latter pathway, superoxide dismutase (SOD) was used to scavenge superoxide [31]. The addition of SOD did not affect the EPR signal intensity of DMPO-OH (data not shown), suggesting that the EPR spectra observed in this study arose from trapping of hydroxyl radicals by DMPO during the decomposition of peroxynitrite. To investigate whether aspirin can inhibit hydroxyl radicals induced by peroxnitrite, different concentration of aspirin were incubated with 1 mM peroxynitrite for 10 min. As shown in Fig. 3, EPR radical spectrum signal heights were significantly inhibited by aspirin (0.25–2 mM) in a concentration-dependent manner. These results represent the first EPR evidence for aspirin to potently inhibit hydroxyl radical formation from peroxynitrite.

To further investigate whether aspirin is a hydroxyl radical scavenger or an inhibitor of its generation, the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$), a typical system for hydroxyl radical generation, was used to test the scavenging effect of aspirin on hydroxyl radicals. As shown in Fig. 4, a spectrum of 1:2:2:1 quartet with splittings at $a_N = a_H = 14.9$ G was observed. These splitting constants and the 1:2:2:1 quartet are indicative of the DMPO-OH adduct [32]. The addition of 2 mM aspirin, significantly decreased the DMPO-OH adduct signal by 51.2%, compared with 25.4% and 82.5% caused by 2 and 10 mM mannitol (a known hydroxyl radical scavenger) respectively [33], indicating aspirin is a more potent hydroxyl radical scavenger than mannitol. Taken together, these results may suggest that the inhibitory effects of aspirin on peroxynitrite-induced DNA damage could be attributed to the scavenging of hydroxyl radicals from peroxynitrite.

In summary, this study demonstrates for the first time that aspirin at pharmacologically relevant concentrations is able to inhibit peroxynitrite-induced DNA cleavage. Accumulating evidence suggests that peroxynitrite is critically involved in the development of various types of cancers, in particular colorectal carcinoma, and induction of DNA damage by peroxynitrite is suggested to be a critical event, leading to multistage carcinogenesis [34,35]. Accordingly, the ability of aspirin to inhibit peroxynitrite-induced DNA damage and hydroxyl radical formation as dem-

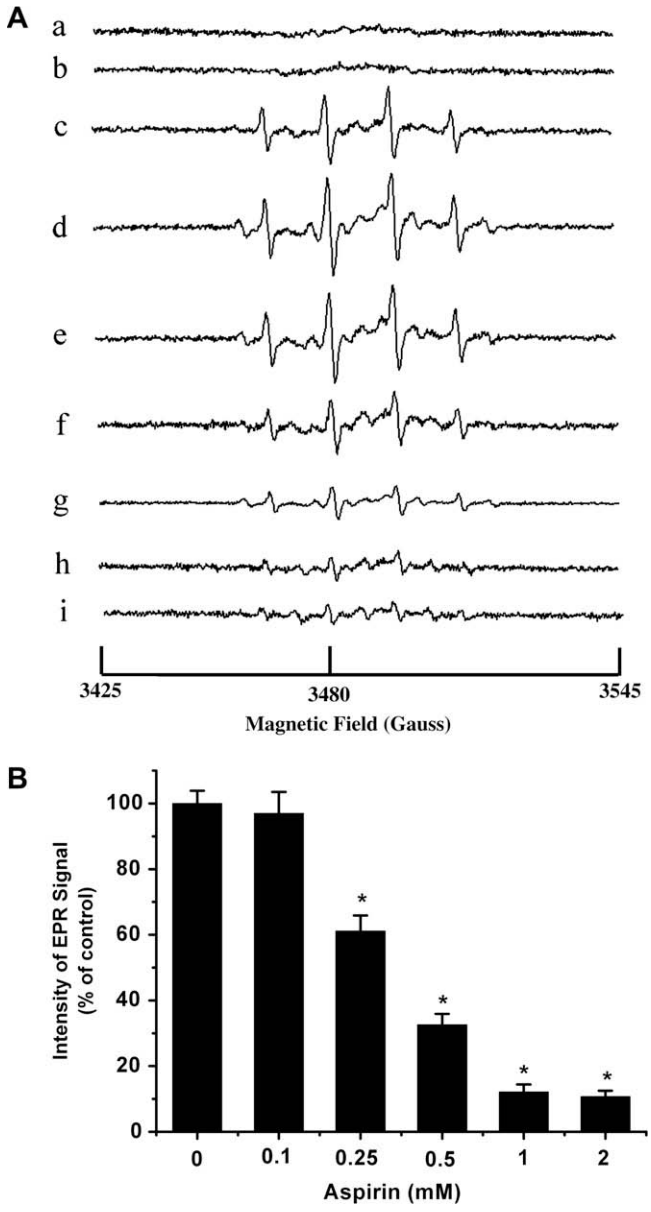

Fig. 3. Effect of aspirin on hydroxyl radical formation from peroxynitrite. EPR spectroscopy in combination with DMPO and peroxynitrite were used to examine the free radical scavenging ability of aspirin. (A) Line a, without peroxynitrite and aspirin; line b, 2 mM aspirin only; line c, 100 μM peroxynitrite only; line d, 1000 μM peroxynitrite only; line e, 0.1 mM aspirin + 1000 μM peroxynitrite; line f, 0.25 mM aspirin + 1000 μM peroxynitrite; line g, 0.5 mM aspirin + 1000 μM peroxynitrite; line h, 1 mM aspirin + 1000 μM peroxynitrite; line i, 2 mM aspirin + 1000 μM peroxynitrite. EPR measurement conditions were as described in Materials and methods. Data represent similar results from three independent experiments. (B) Signal intensity at 3480 G was expressed as mean ± SD from three separate experiments (*$p < 0.05$ versus control).

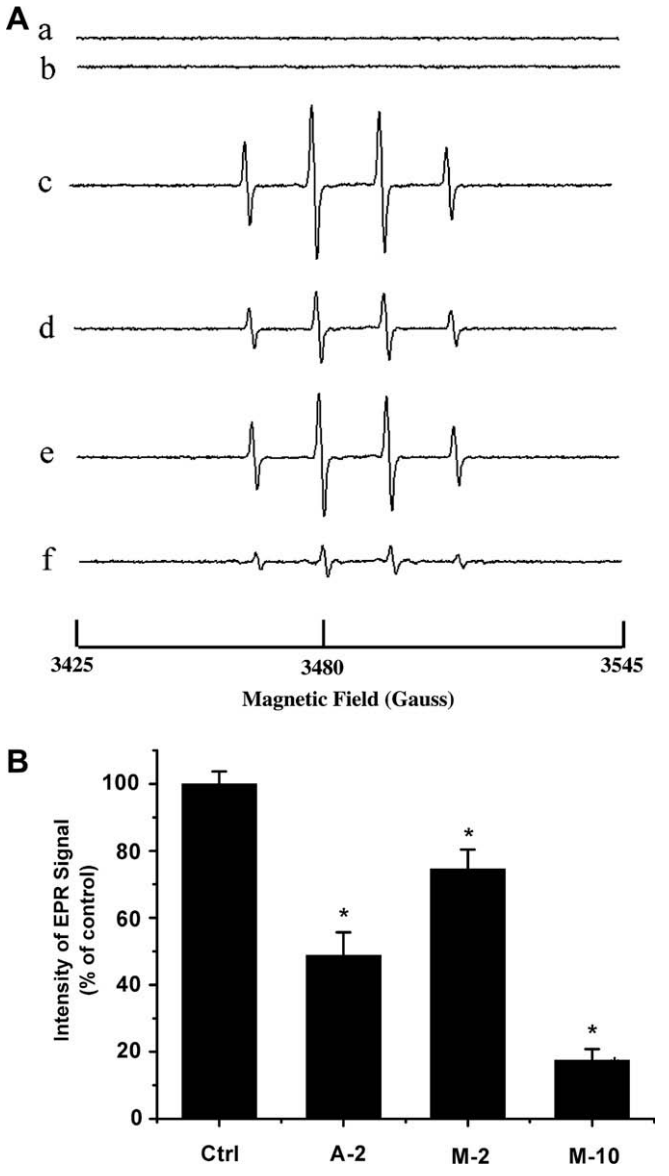

Fig. 4. Scavenging effect of aspirin on hydroxyl radicals generated from the Fenton reaction. EPR spectra of DMPO-OH observed during the reaction of 250 μM ferrous sulfate and 250 μM H_2O_2 containing 80 mM DMPO, in the presence or absence of aspirin. (A) Line a, 80 mM DMPO alone; line b, 2 mM aspirin alone; line c, 250 μM $FeSO_4$ and 250 μM H_2O_2; line d, 250 μM $FeSO_4$ and 250 μM H_2O_2, and 2 mM aspirin; line e, 250 μM $FeSO_4$ and 250 μM H_2O_2, and 2 mM mannitol; line f, 250 μM $FeSO_4$ and 250 μM H_2O_2, and 10 mM mannitol. EPR measurement conditions were as described in Materials and methods. Data represent similar results from three independent experiments. (B) Signal intensity at 3480 G was expressed as mean ± SD from three separate experiments. Ctrl: 250 μM $FeSO_4$ and 250 μM H_2O_2; A-2: 250 μM $FeSO_4$ and 250 μM H_2O_2, and 2 mM aspirin; M-2: 250 μM $FeSO_4$ and 250 μM H_2O_2, and 2 mM Mannitol; M-10: 250 μM $FeSO_4$ and 250 μM H_2O_2, and 10 mM Mannitol. (*$p < 0.05$ versus control).

onstrated in the present study may have implications for cancer intervention by this commonly used anti-inflammatory agent.

Acknowledgments

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