Free Rad. Res. Comms., Vol. 9, No. 3-6, pp. 337-342
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SPIN TRAPPING OF IBUPROFEN RADICALS: EVIDENCE THAT IBUPROFEN IS A HYDROXYL RADICAL SCAVENGER

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The purpose of this study was to use electron paramagnetic resonance (EPR) spectroscopy to determine if ibuprofen, [2-(4-isobutylphenyl) propanoic acid], a potent nonsterodial anti-inflammatory agent, could modify hydroxyl radicals generation *in vitro*. Ibuprofen (IBU; 0.1–50 mM) in water or water alone was added to EPR tubes containing ferrous sulfate (0.5–2.0 mM), and either 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; 40 mM) or α-phenyl N-*tert*-butyl nitrone (PBN; 48 mM). Hydrogen peroxide (1 mM) was added to inititate the Fenton reaction, and the systems were then analyzed by EPR spectroscopy to determine the type and relative quantity of free radical(s) produced. IBU caused a dose-dependent decrease of signal intensity of the hydroxyl radical adduct of DMPO (DMPO-OH) which is an indication that IBU either scavenges the hydroxyl radical and/or chelates iron. In addition, other radicals (presumably IBU radicals) produced in these systems were trapped by both DMPO (a_N = 16.1 G, a_β^H = 24.0 G) and PBN (a_N = 15.7 G, a_β^H = 4.4 G and a_N = 17.0 G, a_β^H = 2.1 G). The signal height of these IBU radicals increased in systems containing ferrous sulfate (1 mM), hydrogen peroxide (1 mM), PBN (48 mM), and increasing IBU concentrations. Therefore, we conclude that IBU scavenges the hydroxyl radical. If IBU chelated iron, then less hydroxyl radicals would be generated, less IBU radicals formed and the signal height of IBU radicals trapped by PBN would have decreased. However, these data do not fully exclude the possiblity that IBU may, to some extent, also chelate iron. Scavenging of hydroxyl radicals may be one of the mechanisms responsible for the beneficial action of IBU during the management of several rheumatic diseases. However, the IBU radicals produced when IBU scavenges hydroxyl radicals are reactive, and may be associated with the reported toxicity of this therapeutic agent.

KEY WORDS: Ibuprofen, spin trapping, electron paramagnetic resonance, 5,5-dimethyl-1-pyrroline-N-oxide, α-phenyl N-*tert*-butyl nitrone.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to suppress the signs and symptoms of inflammation.[1,2] It has been proposed[3] that the basis of their actions are related to their ability to inhibit the synthesis of prostaglandins; however, this hypothesis may not account for all of the effects of NSAIDs at all dosages. It has been reported [3] that NSAIDs may possess other actions besides inhibiting cyclo-oxygenase. These actions include inhibition of platelet and neutrophil aggregation and disruption of a variety of processes at the plasmalemma that regulate the generation of intracellular signalling.[3] However, additional mechanisms of action of NSAIDs have not been completely elucidated.

Ibuprofen (Motrin, Rufen), [2-(4-isobutylphenyl)propanoic acid], is a NSAID that is widely prescribed for the treatment of a variety of rheumatic disorders, especially in patients with rheumatoid arthritis and osteo-arthritis. It is also available at lower dosages over the counter as Advil and Nuprin. We propose that an additional

anti-inflammatory action of ibuprofen (IBU) is its ability to alter the generation of hydroxyl radicals. Evidence supporting the contribution of reactive oxygen species, like hydroxyl radicals, in diseases associated with inflammatory have been reviewed by Southorn and Powis.[4] Highly reactive hydroxyl radicals can cause tissue injury either directly, or indirectly by initiating lipid peroxidation.[5]

Therefore, the pupose of this study was to determine the antioxidant capacity of IBU by combining electron paramagnetic reasonance (EPR) spectroscopy and spin trapping techniques. Hydroxyl radicals generated *in vitro* by the Fenton reaction react with 5,5-dimethyl pyrroline-N-oxide (DMPO) to produce the stable paramagentic hydroxyl radical adduct of DMPO (DMPO-OH) that has a characteristic EPR spectrum. In this study, we determined the signal intensity of DMPO-OH in Fenton reactions containing different concentrations of IBU. A decrease in signal intensity of DMPO-OH may be associated with the ability of IBU to scavenge hydroxyl radicals directly or cheltate iron, and thus reduce hydroxyl radical formation via Fenton -type chemistry. DMPO and alpha-phenyl N-*tert*-butyl nitrone (PBN) both have the capacity to also trap carbon-centered radicals, generating paramagnetic adducts that can be detected using EPR spectroscopy. The presence of carbon-centered radicals would suggest an attack on IBU by hydroxyl radicals, and an increased signal height of these carbon-centered radicals at higher concentrations of IBU would suggest the ability of IBU to scavenge hydroxyl radicals.

MATERIALS AND METHODS

The test system contained ferrous sulfate (0.5–2 mM), IBU (0.1–50 mM) and either DMPO (40 mM) or PBN (48 mM). One minute after the addition of hydrogen peroxide, the EPR spectra were recorded on an IBM/Bruker ESP 300C with an ESP 1600 data system using an X-band klystron, 100 kHz modulation, a 10 inch magnet and an ER4102ST cavity. The following spectrometer settings were used: scan width, 100 gauss (G); time constant, 327.68 msec; modulation amplitude, 0.975 G; signal channel gain, 5.0 e4 or 1.0 e5 and microwave power, 19.8 mW. DMPO and PBN were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Sigma Chemical Co. (St

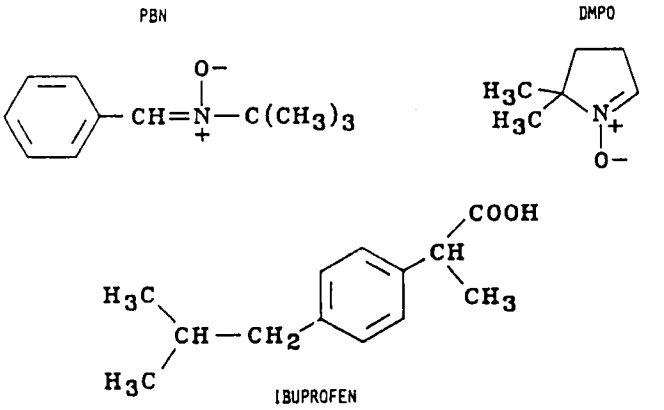

FIGURE 1 Structures of DMPO, PBN and ibuprofen

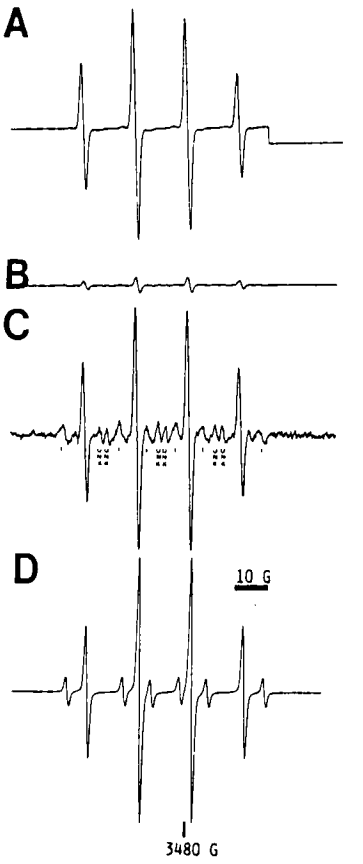

FIGURE 2 EPR spectra of Fenton systems containing DMPO. Hydroxyl radicals were produced, as described in the Methods section, in the presence of DMPO (Panel A) or presence of DMPO and ibuprofen (50 mM) (Panel B). Panel C was obtained after the scale of Panel B was increased sixteen-fold; this allowed for visulatization of another adduct of DMPO, designated I, and unknown signals, designated UNK. Panel D is a simulation of DMPO-OH and I.

Louis, MO), respectively (Figure 1). IBU was obtained from the Upjohn Co. (Kalamazoo, MI) (Figure 1). All other chemicals were of reagent grade quality.

RESULTS

EPR spectra, characteristics of the hydroxyl radical adduct of DMPO (DMPO-OH), were obtained by mixing ferrous sulfate, DMPO and hydrogen peroxide (Figure 2). Addition of IBU to this system reduced the DMPO-OH signal height in a concentration-dependent fashion (Figure 4 and Table 1). Besides trapping hydroxyl radicals ($a_N = a_\beta^H = 14.9\,G$) another radical ($a_N = \alpha_\beta^H = 24.0\,G$) was also trapped by DMPO in systems containing higher concentrations of IBU (Figure 2).

No signals were detected after hydrogen peroxide was added to systems containing

TABLE 1

Peak height (mm) of the hydroxyl radical adduct of DMPO in the presence of ferrous sulfate ($FeSO_4$; 0.5–2 mM), hydrogen peroxide (1 mM), DMPO (40 mM) and different concentrations of ibuprofen. Data (mean ± SEM, n = 6) were normalized to a gain of 5.0 e 4 and scale of 16.

	$FeSO_4$ 0.5 mM	$FeSO_4$ 1.0 mM	$FeSO_4$ 2.0 mM
No Ibuprofen	187 ± 9	297 ± 12	441 ± 20
Ibuprofen			
0.5 mM	122 ± 6	308 ± 15	466 ± 20
2.5 mM	136 ± 7	277 ± 13	392 ± 15
5.0 mM	91 ± 5	199 ± 12	298 ± 10
50.0 mM	15 ± 2	19 ± 1	14 ± 2

ferrous sulfate either in the presence and absence of PBN (Figure 3). However, two signals (a_N = 15.7 G, a_β^H = 4.4 G and a_N = 17.0 G, a_β^H = 2.1 G) were observed when IBU was incorporated into systems containing ferrous sulfate, hydrogen peroxide and PBN (Figure 3). Increasing the concentration of IBU by ten-fold resulted in a two-fold increase in the height of the first peak of these PBN-radical signals (Table 2).

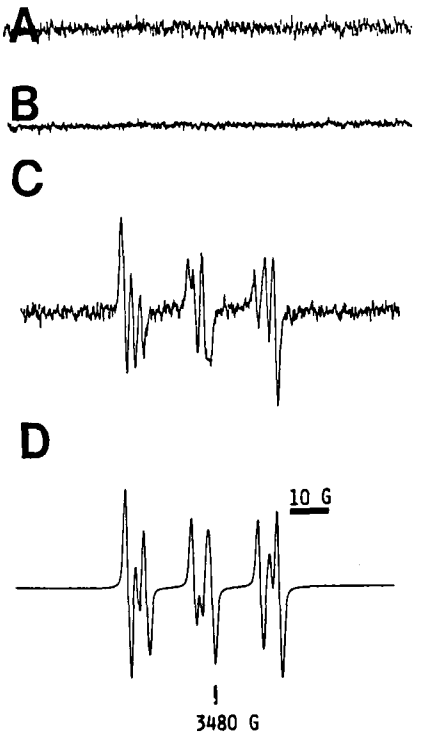

FIGURE 3 EPR spectra of systems containing PBN. Hydroxyl radicals were produced, as described in the Methods section, in the presence of PBN alone (Panel A) or absence of PBN but presence of ibuprofen (50 mM) (Panel B) or presence of both PBN and ibuprofen (Panel C). Panel D is a simulation of the two radical species observed in panel C.

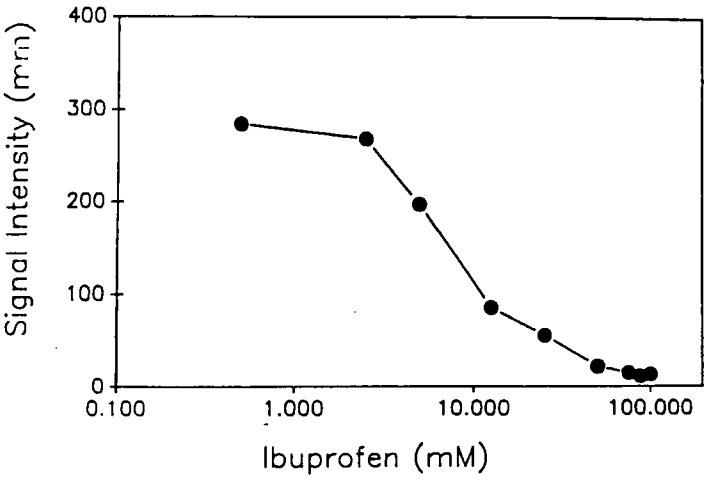

FIGURE 4. A representative experiment on the effect of increasing concentrations of ibuprofen on signal intensity of DMPO-OH.

DISCUSSION

We used EPR spectroscopy to determine if IBU alters the ability of DMPO to trap hydroxyl radicals generated in a Fenton-type reaction. Since signal height is proportional to the number of free radicals produced during the reaction, and IBU reduced the height of the hydroxyl radical adduct of DMPO (DMPO–OH) (Figure 2 and Table 1), we concluded that IBU may scavenge hydroxyl radicals and/or affect their production. Blasig *et al.*[6] have suggested that reduction of the signal height of DMPO–OH by a xenobiotic indicated that hydroxyl radicals were scavenged. However, chelation of iron by a xenobiotic will reduce hydroxyl radical production by interfering with the Fenton reaction, resulting in a decreased production of hydroxyl radicals and, hence, fewer radicals being trapped by DMPO.

To answer the question concerning the ability of IBU to scavenge hydroxyl radicals or chelate iron, we replaced DMPO with PBN in our Fenton systems. We hypothesized that if IBU scavenged the hydroxyl radical, then carbon-centered free radicals would be generated by hydroxyl radical attack on IBU. To support this hypothesis, it was observed that in a Fenton system containing DMPO and high concentrations of IBU, DMPO adducts of weak intensity were formed by reaction of DMPO with unidentified free radicals and these EPR signals were different from DMPO-OH (Figure 2). In Fenton systems containing PBN and IBU, two radicals were trapped

TABLE 2

Peak height (mm) of the downfield peak of ibuprofen radical adduct of PBN produced in the presence of ferrous sulfate (1 mM), hydrogen peroxide (1 mM) and PBN (48 mM). Data (mean ± SEM, n = 6) was normalized to a gain of 1.0 e 5 and scale of 16.

Ibuprofen (5 mM)	Ibuprofen (50 mM)
54 ± 3	118 ± 5

by PBN (Figure 3). These may be the same or different radicals which was trapped by DMPO (Figure 2). Since the signal height of the PBN adducts increased with higher concentrations of IBU (Table 2), we concluded that IBU predominately scavenged the hydroxyl radical. If IBU was exerting its effect by chelating iron, then the signal height of the PBN adducts should have decreased. That is, with less free iron available for the Fenton reaction, fewer hydroxyl radicals would have been produced and, as a consequence, fewer IBU radicals would have been generated by reaction of IBU with hydroxyl radicals.

This study demonstrates that ibuprofen scavenges hydroxyl radicals. Since the hydroxyl radical has been implicated in the pathophysiological abnormalities associated with many diseases (i.e. inflammation, rheumatoid arthritis, osteo-arthritis)[4] treated by IBU, the mechanism responsible for the beneficial effects of IBU may include its ability to scavenge hydroxyl radicals. In view of the fact that the reaction of IBU with hydroxyl radicals (and perhaps other free radicals) produces reactive IBU radicals, it is possible that such radicals may be involved in the reported toxicity (hematologic effects, acute renal failure, interstitial nephritis and nephrotic syndrome) of ibuprofen.[7,8,9]

Acknowledgement

The authors wish to thank Dr. J. Lee Poyer for intellectual contribution and Ms. Cindy Williams for secretarial assistance in the preparation of this manuscript. This work was supported in part by NIH grants GM36512 and T32-GM08237.

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Accepted by Prof. E.G. Janzen