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SUPEROXIDE, HYDROXYL AND FATTY ACID RADICAL SCAVENGING BY AMINOSALICYLATES

DIRECT EVALUATION WITH ELECTRON SPIN RESONANCE SPECTROSCOPY

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Abstract—Reactive oxygen radicals such as superoxide and hydroxyl radicals, as well as intermediate unsaturated fatty acid radicals, have been proposed as playing an important role in various diseases including inflammatory bowel disease (IBD). In this study we evaluated radical scavenger properties of aminosalicylates used in the therapy of IBD using spin trapped electron spin resonance spectroscopy. 5-Aminosalicylic acid (5-ASA), 4-aminosalicylic acid and olsalazine had superoxide radical scavenger properties (IC_{50} = 0.4, 0.4 and 1.0 mM, respectively). 5-ASA and benzalazine also inhibited hydroxyl radicals (IC_{50} = 6.5 mM). Fatty acid radicals were not inhibited by aminosalicylates. Our results support the hypothesis that therapeutically active compounds may be oxygen radical scavengers and that fatty acid radical scavenging has to be performed by drugs other than aminosalicylates.

Reactive oxygen radicals such as superoxide ($O_2^{\cdot}$) and hydroxyl ($OH^{\cdot}$) radicals have been proposed as playing an important role in the cell damage involved in various diseases including ischemia–reperfusion injuries, rheumatoid arthritis and inflammatory bowel disease (IBD§) [1–7]. Intermediate radicals from unsaturated fatty acids formed by autooxidation or through the lipoxygenase reaction have also been suggested as being involved in cell damage [8, 9]. Radical scavenging, therefore, has been considered a putative mechanism of therapeutic action [10–13]. From studies using indirect radical detection methods such as cytochrome *c* reduction, decarboxylation of benzoate or chemoluminescence it has been hypothesized that compounds active in the treatment of IBD such as 5-ASA may be radical scavengers [1, 5, 10–13]. To study this hypothesis in more detail we compared the radical scavenger properties (superoxide, hydroxyl and linoleate radicals) of therapeutically active compounds such as 5-ASA [14], 4-ASA, SAZ, ADS and benzalazine using systems with defined radical production, and direct evaluation with ESR. ADS has been introduced recently into the treatment of IBD [15]. 4-ASA and benzalazine were found to be effective in preliminary clinical trials [16, 17].

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§ Abbreviations: ADS, azodisalicylate (olsalazine); 5-ASA, 5-aminosalicylic acid; N-Ac-ASA, *N*-acetyl-aminosalicylic acid; SAZ, sulfasalazine; SP, sulfapyridine; NGDA, nordihydroguaretic acid; ESR, electron spin resonance; DMPO, 5-5′dimethyl-pyrroline *N*-oxide; DBNBS, 3,5′dibromo 4-nitrosobenzene sulfonic acid; IBD, inflammatory bowel disease; SOD, superoxide dismutase; DMSO, dimethyl sulfoxide.

MATERIALS AND METHODS

SAZ, SP, 5-ASA and ADS were gifts from the Deutsche Pharmacia GmbH (Ratingen, F.R.G.): 4-ASA and N-Ac-ASA acid were supplied by courtesy of Dr U. Klotz (Stuttgart, F.R.G.). NGDA, DBNBS, DMPO, sodium linoleate and soybean lipoxygenase were of analytical grade and purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Superoxide, hydroxyl and linoleate radicals, the latter representing unsaturated intermediate fatty acid radicals, [8], were generated as described below. As these radicals are extremely short-lived, stabilization with specific spin traps such as DBNBS or DMPO is necessary to detect measurable signals in the magnetic field of ESR spectroscopy [18, 19]. Superoxide radicals were produced according to Hyland and Auclair [20] using 0.5 M NaOH in DMSO (1%, v/v) with DBNBS (1.9 mM) as the spin trap. Superoxide production was further quantitated with the SOD-dependent cytochrome *c* reduction (molar extinction coefficient 21.4 $mM^{-1}cm^{-1}$) [20] and correlated with the ESR peak heights (Table 1). The detection limit using spin trap ESR under these conditions was 0.02 absorption units at 550 mm (0.09 μM $O_2^{\cdot}$). Hydroxyl radicals were produced by a modified Fenton reaction according to Ahnfelt-Ronne *et al.* [12] with $FeSO_4$ (5 μM), H_2O_2 (44 μM) and EDTA (22 μM) in a sodium phosphate buffer (0.1 M, pH 7.2) with DMPO (0.09 M) as the spin trap. Linoleate radicals were produced by the soybean lipoxygenase reaction [21]: sodium linoleate 1.1 mM and lipoxygenase 3.3×10^6 U/mL in 0.1 M borate buffer (pH 9.0) with DBNBS (2.5 mM) as the spin trap. All inhibitors were added prior to the corresponding compounds in concentrations as indicated. The drugs were prepared in aqueous solutions adjusted to the corresponding pH; 5-ASA

Table 1. Superoxide radical quantitation: correlation of ESR peak heights and cytochrome c reduction (E_{550})

Conditions (mL DMSO/OH)	ESR peak height ($\times 10^4$) (arbitrary units)	E_{550}
0.8	3.78 ± 0.15	0.14 ± 0.012
0.6	1.41 ± 0.21	0.04 ± 0.005
0.4	0.36 ± 0.03	0.02 ± 0.005
0.2	ND	<0.01

Values are means ± SEM; N = 5; the total assay volume was 1 mL.

The SOD-inhibitable (200 μg/mL) cytochrome c reduction was 60 ± 5%; $O_2^{\cdot}$ radical concentrations with 0.8 mL DMSO/OH were 0.39 μM; ND, no ESR signal detectable.

(20 mM) was dissolved in 0.1 M NaCl pH 9.0 prior to use. Potential splitting of the azodrugs to their 5-ASA components and preservation of the azobonds were monitored by UV spectroscopy and HPLC [22]. ESR measurements were performed in a glass capillary at room temperature with Bruker ESP 300 equipment using a microwave power of 10 dB, a modulation frequency of 100 kHz and modulation amplitude of 1 G. All measurements were performed 1 hr after the addition of the spin traps, as signal intensities were maximal at this time with a slow decrease thereafter. For quantitation, peak heights were expressed in arbitrary units. In the inhibition experiments, drug effects were measured as the relative percentages of peak heights compared to incubations with no drugs. All experiments were repeated four to six times (N = 4–6) and expressed as means ± SEM. The coefficient of variation of the ESR peak heights was 13.5% for the DBNBS/O_2 and 12.0% for the DMPO/OH spin adduct. The coefficient of variation of the SOD-dependent cytochrome c reduction was 10.5% (N = 16).

RESULTS AND DISCUSSION

5-ASA concentration-dependently suppressed superoxide–DBNBS-induced ESR signals (Fig. 1). The ESR spectrum of the stable radical product formed by the DBNBS spin trap features a prominent G = 2.0066 triplet originating from a nitrogen hyperfine coupling of a_N = 12.63 G and an unresolved proton coupling with a_H = 0.71 G [18]. Quantitation of superoxide production under these conditions yielded 390.0 nmol $O_2^{\cdot}$/mL using cytochrome c reduction as a second method (Table 1). As seen from this figure, 50% reduction of the peak heights occurred at 0.4 mM; above 2 mM there was almost complete suppression of the ESR signals. Similar patterns of signal suppression and comparable IC_{50} were observed with 4-ASA and ADS but not with SP, SAZ, benzalazine or N-Ac-ASA (Table 2). Hydroxyl–DMPO-induced ESR signals, consisting of a dominant quartet signal with an intensity distribution of 1:2:2:1 due to equivalent nitrogen

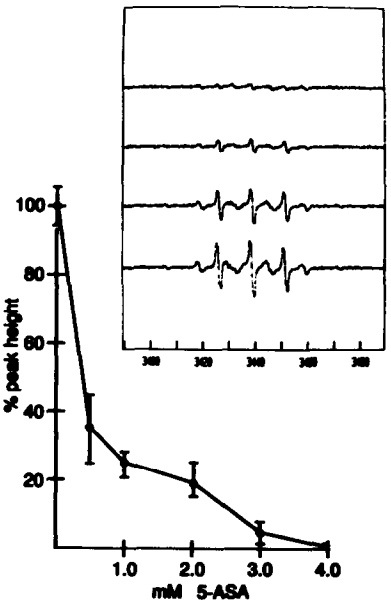

Fig. 1. Inhibition of $O_2^{\cdot}$–DBNBS-induced ESR signals (relative peak heights) by 5-ASA. Inset: ESR spectrum from a representative incubation. The lines represent (from bottom to top) spectra with no inhibitor (bottom) and with 5-ASA, 0.5, 1.0 and 2 mM.

Table 2. Comparison of inhibition of superoxide radical production by aminosalicylates and SP in the DMSO/OH system

	IC_{50} (mM)
5-ASA	0.4 ± 0.03
4-ASA	0.4 ± 0.03
SAZ	—
ADS	1.0 ± 0.02
Benzalazine	—
SP	—
N-Ac-ASA	—

Spin trap: 1 mM DBNBS; values are means ± SEM; N = 6; —, no inhibition (maximum drug concentrations 10 mM), concentrations ≥10 mM: interference with the ESR signals.

and proton hyperfine coupling constants of a_N = a_H = 15 G [19], were suppressed concentration-dependently by 5-ASA (Fig. 2) (IC_{50} = 6.5 mM) and benzalazine (not shown) (IC_{50} = 6.5 mM), but not by any of the other compounds. Linoleate radical–DBNBS-induced ESR signals (a_N = 14.6 G, a_H = 12.3 G) were inhibited by NGDA, a known lipoxygenase inhibitor, but not by 5-ASA (Fig. 3) or the other derivatives (not shown). During incubation of ADS and benzalazine with their corresponding radicals, small amounts (0.8%) of 5-ASA were detected by HPLC. There was also a

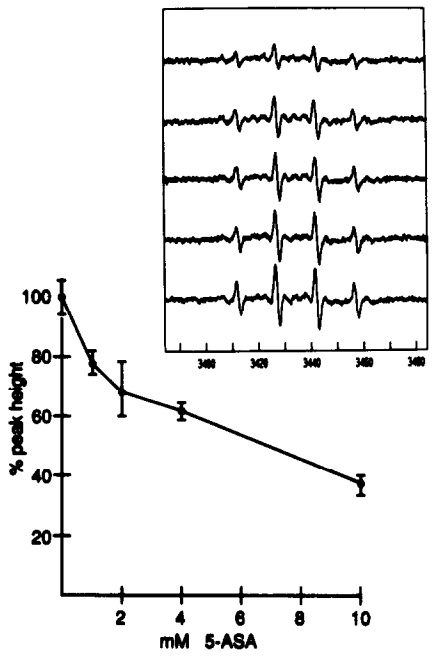

Fig. 2. Inhibition of $O_2^{\cdot}$–DPMO-induced ESR signals (relative peak heights) by 5-ASA. Inset: ESR spectum from a representative incubation. The lines represent (from bottom to top) spectra with no inhibitors (bottom) and with 5-ASA, 1.0, 2.0, 3.0 and 10 mM.

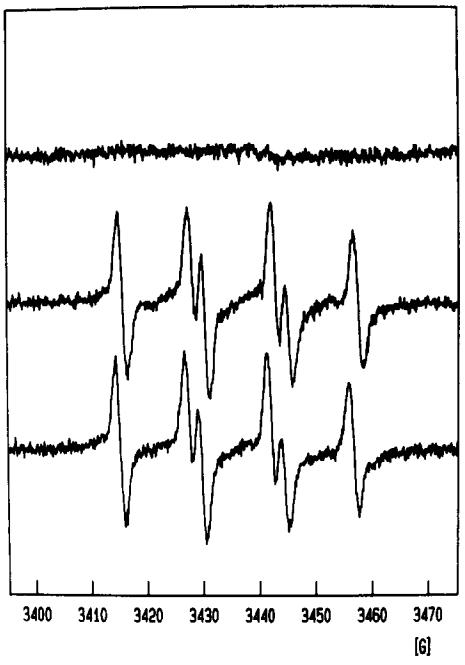

Fig. 3. Comparison of effects of NGDA and 5-ASA acid on linoleate radical–DBNBS-induced ESR signals. The lines represent (from bottom to top) spectra from incubations with no inhibitors (bottom), 2.5 mM 5-ASA and 3.0 mM NGDA.

slight decrease (~20%) in the absorption maxima of ADS and benzalazine.

From this data, using systems with defined radical generation and direct detection by ESR spectroscopy, we have added further support to the hypothesis that compounds active in the treatment of IBD are *in vitro* radical scavengers since we found that 5-ASA, 4-ASA and ADS suppressed superoxide radicals. $OH^{\cdot}$ radicals were suppressed by 5-ASA and benzalazine. We have used spin trap ESR spectroscopy rather than cytochrome *c* reduction, ethylene generation or chemoluminescence as these methods are not entirely specific, interfere with inhibitors or measure additional reactants, e.g. hypochlorite [23]. We were particularly interested in superoxide scavenging as this radical, although only moderately toxic by itself, is the basic $O_2^{\cdot}$ species after the respiratory burst of activated inflammatory cells from which more active compounds such as hydrogen superoxide, hydroxyl radicals (Haber–Weiss reaction) and hypochlorite (myeloperoxidase reaction) are formed [5, 6]. Our observation using ESR of $O_2^{\cdot}$ radical scavenging by 4-ASA differs from the results of other studies in which 4-ASA was demonstrated to be unable to function as a radical scavenger [13]. Possible explanations for this discrepancy may be different radical generation systems [13] and the observation that 4-ASA interferes with the cytochrome *c* assay (personal observations).

$OH^{\cdot}$ radicals were found to be scavenged by 5-ASA and benzalazine, the latter molecule consisting of 5-ASA linked to benzoic acid by an azo bond [7]. It was proposed that $OH^{\cdot}$ scavenging by 5-ASA is not due to a direct interaction but rather is the consequence of its inhibiting due to its iron chelating capacity the iron catalysed Haber–Weiss reaction [24]. The reasons for the higher concentrations of 5-ASA needed to suppress $OH^{\cdot}$ generation compared to superoxide production are not entirely clear but may be ascribed to the different assay conditions including different spin traps, e.g. DBNBS or DMPO, respectively. As seen from HPLC and UV spectroscopy ~80% of the prodrug molecules remain intact, suggesting that radical scavenging may be due mainly to the unsplit compounds. Whether the small amounts of 5-ASA released from their prodrugs during incubation may also contribute to radical scavenging, is possible, but unlikely. Although extrapolations from *in vitro* experiments to the *in vivo* situation are hazardous and a great variety of other factors can contribute to the inflammatory response (e.g. inflammatory mediators etc.), we suggest that radical scavenging by aminosalicylates may also occur in patients with IBD as (1) comparable drug concentrations which inhibited superoxide or hydroxyl radical formation *in vitro* can be expected in the ileal/colonic lumen of patients on these drugs [25] and (2) similar radical concentrations (~1 μM) may be present in inflammatory processes based on data from animal experiments [26, 27]. If radical scavenging is a possible mechanism of therapeutic action, administration of more specific scavengers should lead to decreased inflammation in animal models and the treatment of human disease. Unsaturated fatty acid radical scavenging, on the

other hand, has to be performed with compounds other than 5-ASA, or its derivatives.

In summary, we have demonstrated that 5-ASA, 4-ASA and ADS concentration-dependently inhibit superoxide radical production using a system with defined radical generation and direct evaluation by ESR spectroscopy. $OH^{\cdot}$ radicals were scavenged by 5-ASA and benzalazine but not by the other derivatives. Unsaturated fatty acid (linoleate) radicals were not scavenged by any of these drugs. Our results support the hypothesis that therapeutically active compounds in IBD are *in vitro* reactive oxygen radical scavengers.

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