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Rhein: an anthraquinone that modulates superoxide anion production from human neutrophils

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Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), the active metabolite of diacetylrhein, which has been reported as an effective antirheumatic drug in man, inhibited superoxide anion production from human neutrophils challenged with *N*-formylmethionyl-leucyl-phenylalanine (FMLP: IC50, 2×10^{-5} M) and A23186 (IC50, 10^{-5} M), but not with phorbol myristate acetate. In the same concentration range (10^{-6}–10^{-3} M), the drug did not affect oxy-radical production by a cell-free hypoxanthine-xanthine oxidase system and exerted weak inhibitory effects on FMLP-evoked lysosomal enzyme release. Rhein inhibitory effects on neutrophil functioning may contribute to the overall therapeutic activity of the parent drug, diacetylrhein.

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), an anthraquinone from plants of the genus Cassia, has been described as having weak antibacterial effects and cathartic properties (Kean 1970). It is a derivative of sennosides and these anthraquinones have been demonstrated to stimulate colonic fluid and electrolyte secretion and PGE_2 formation in the rat colon, in-vivo (Beubler & Kollar 1985).

The drug was reported to interfere with mitochondrial electron transport and to inhibit flavoproteins concerned with redox reactions of pyridine nucleotides (Kean 1968, 1970; Kean et al 1971). Furthermore, Raimondi et al (1982) showed that rhein, in the concentration range 10^{-5}–10^{-4} M, inhibited some proteases in-vitro, with bovine pancreatic carboxypeptidase A and porcine pancreatic elastase being the most sensitive enzymes.

Rhein is the active metabolite of diacetylrhein (DAR: 1,8-diacetoxy-9, 10-dioxo-dihydroanthracene-3-carboxylic acid), which some authors have reported to exert anti-inflammatory effects in in-vivo animal tests and to display antirheumatic activity in man (Kay et al 1980; Neuman 1980; Pomarelli et al 1980). Diacetylrhein, in in-vivo experiments, and rhein, in in-vitro experiments, did not inhibit prostaglandin synthesis in inflammatory exudates and in guinea-pig lung homogenates that had been incubated with arachidonic acid and challenged with rhein in the concentration range $3{\cdot}2 \times 10^{-6}$–$3{\cdot}2 \times 10^{-4}$ M (Pomarelli et al 1980; Franchi-Micheli et al 1983).

Human neutrophils, a cell type that plays a key role in inflammatory processes, are known to degranulate, aggregate and experience a 'respiratory burst' (which is characterized by the generation of reactive oxygen-derived free radicals), in response to a variety of soluble and insoluble agents. An NAD(P)H-dependent flavoprotein has been implicated in superoxide anion ($O_2^{\cdot-}$) production from human neutrophils (Babior 1984), whereas tissue destruction in chronic disorders (such as emphysema and arthritis) has been related to the release of lysosomal proteases, and particularly to the release of the azurophil granule enzyme, elastase (Saklatvala 1977; Werb et al 1982). Because of the efficacy of DAR in-vivo and rhein's ability to inhibit protease enzymes and flavoproteins in-vitro, we decided to verify whether or not it could interfere with $O_2^{\cdot-}$ production and lysosomal enzyme release from human neutrophils.

The effects of rhein were evaluated in cells which had been activated in-vitro by different stimuli: a chemotactic peptide (*N*-formylmethionyl-leucyl-phenylalanine: FMLP), a calcium ionophore (A23187) and an activator of protein-kinase C (phorbol myristate acetate, PMA: Nishizuka 1984).

Materials and methods

Neutrophils were isolated from healthy adult volunteers by standard techniques of dextran sedimentation (dextran T500, Pharmacia), Ficoll-Paque (Pharmacia) gradient centrifugation and hypotonic lysis of erythrocytes, as previously described (Fantozzi et al 1986).

Neutrophils were suspended in a buffered salt solution (mM: NaCl 138, KCl 2·7, Na_2HPO_4 8·1, KH_2PO_4 1·5, $MgCl_2$ 1, $CaCl_2$ 2, pH 7·4) supplemented with glucose (1 mg mL^{-1}) and human serum albumin (1 mg mL^{-1}: Behringwerke). When indicated, experiments were carried out by using a calcium-free medium containing EGTA [1 mM; ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid: Sigma].

Cells were treated with cytochalasin B (5 μg mL^{-1}; Aldrich) for 5 min, before exposure to rhein (kindly supplied by Istituto Gentili S.p.A., Pisa, Italy, as sodium salt). After incubation with rhein, neutrophils were challenged with different stimuli (FMLP: Serva; A23187: Calbiochem; PMA: Sigma) at 37 °C.

$O_2^{\cdot-}$ production was continuously monitored spectrophotometrically at 37 °C, by determining superoxide dismutase (SOD: Boehringer-Mannheim)-inhibitable

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cytochrome C (Boehringer-Mannheim) reduction as previously described (Fantozzi et al 1986) and expressed as nmoles cytochrome C reduced $min^{-1}/10^6$ cells (Smolen et al 1981).

β-Glucuronidase (substrate: phenolphthalein glucuronic acid, Sigma) and myeloperoxidase (substrate: *o*-dianisidine; Sigma) were determined as reported by Fantozzi et al (1986). Elastase (substrate: methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide; Sigma) was measured according to Hojima et al (1983).

Xanthine oxidase (XOD) and hypoxanthine (HPX) were from Boehringer-Mannheim and Sigma, respectively.

Rhein inhibitory effects were expressed as percentage inhibition of the values obtained with the stimulus alone.

Results

Rhein, in the concentration range 10^{-6}–10^{-3} M, inhibited $O_2^{\cdot-}$ production from FMLP-activated human neutrophils: the effect was dose-dependent (IC50 = 2×10^{-5} M) and maximal inhibition was achieved at $1{\cdot}7 \times 10^{-4}$ M rhein (Fig. 1A).

In the experiments depicted in Fig. 1A, neutrophils were preincubated with rhein for 10 min; however, $O_2^{\cdot-}$ production was inhibited to the same extent even when cells had been treated with the drug for different times (up to 30 min). Similar inhibitory effects were recorded by adding rhein immediately before the stimulus.

Rhein, even at the highest concentration ($3{\cdot}5 \times 10^{-4}$ M), did not affect oxy-radical production by a cell-free XOD (0·2 U mL^{-1})/HPX (10^{-4} M) system.

When the experiments were performed by incubating human neutrophils in a calcium-free medium containing EGTA 1 mM, rhein still affected $O_2^{\cdot-}$ production from FMLP-activated neutrophils. The effect was dose-dependent and inhibition was similar to that recorded in the presence of calcium in the medium (Fig. 1B).

Rhein inhibited $O_2^{\cdot-}$ production from A23187-stimulated neutrophils in a dose-dependent manner (IC50 = 10^{-5} M). Maximal inhibitory effects were similar to those obtained in FMLP-activated cells (Fig. 2). When neutrophils were challenged with PMA, rhein was devoid of any inhibitory action, even at the concentration of $3{\cdot}5 \times 10^{-4}$ M, which exerted maximal effects on $O_2^{\cdot-}$ production from FMLP- and A23187-activated human neutrophils (Fig. 2). In the same concentration range, rhein not only inhibited $O_2^{\cdot-}$ production, but also affected lysosomal enzyme (e.g., β-glucuronidase, elastase, myeloperoxidase) release from FMLP-stimulated cells. The extent of inhibition was significantly less than that recorded by evaluating $O_2^{\cdot-}$ production; a 30% inhibition of β-glucuronidase, myeloperoxidase and elastase release was obtained at the rhein concentration of 7×10^{-5} M.

Higher concentrations of rhein directly inhibited the enzyme activities that were evaluated in the supernatants of FMLP-challenged human neutrophil suspensions: a 50% inhibition of elastase activity was measured at the rhein concentration of $3{\cdot}5 \times 10^{-4}$ M.

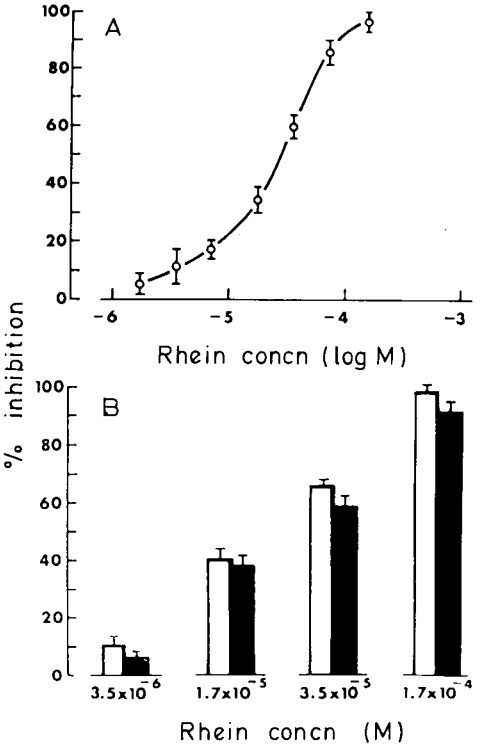

FIG. 1. Effect of rhein on FMLP-evoked superoxide anion production from human neutrophils. (A) Cells stimulated by FMLP 10^{-7} M produced 4·28 ± 0·5 nmol cytochrome C reduced $min^{-1}/10^6$ cells (n = 7). Values obtained in the absence of rhein were taken as 100%. Results are the means ± s.e.m. of 7 experiments. (B) □: cells suspended in a medium containing 1 mM calcium produced 5·37 ± 0·1 nmol cytochrome C reduced $min^{-1}/10^6$ cells. ■: cells incubated in a calcium-free medium plus 1 mM EGTA produced 2·26 ± 0·1 nmol cytochrome C reduced $min^{-1}/10^6$ cells. Values are the means ± s.e.m. of 3 experiments.

Discussion

The results presented here demonstrate that rhein inhibited $O_2^{\cdot-}$ production from human neutrophils, without exerting scavenging effects in a cell-free XOD/HPX system.

Rhein displayed a stimulus-selectivity, since it inhibited with similar potencies $O_2^{\cdot-}$ production from human neutrophils challenged with FMLP or A23187, but not with PMA. The reasons for this stimulus-selectivity are not clear at present, but might be related to the different abilities of these agents to affect calcium-dependent steps that underlie neutrophil activation, since calcium is widely believed to represent a major mediator of neutrophil activation (Naccache et al 1985; Westwick & Poll 1986).

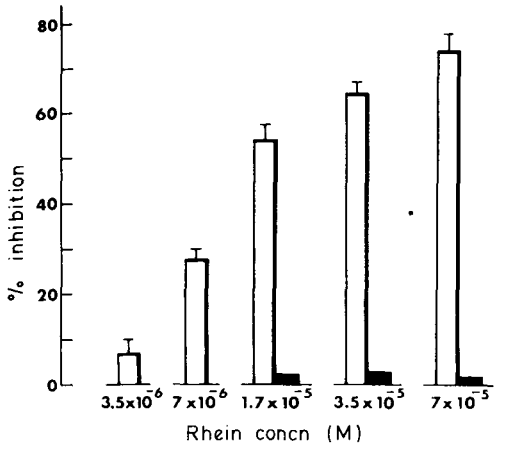

FIG. 2. Effect of rhein on superoxide anion production from human neutrophils challenged with A23187 or PMA. Cells stimulated by A23187 10^{-5} M (□) produced 1·63 ± 0·1 nmol cytochrome C reduced $min^{-1}/10^6$ cells (n = 3). Cells challenged with PMA 50 μg mL^{-1} (■) produced 9·43 ± 0·2 nmol cytochrome C reduced $min^{-1}/10^6$ cells (n = 3). Values obtained in the absence of rhein were taken as 100%. Results are the means ± s.e.m. of 3 experiments.

Actually, FMLP was capable of evoking $O_2^{\cdot-}$ production even in the presence of EGTA, while A23187 greatly depended on extracellular calcium to induce $O_2^{\cdot-}$ production (Pozzan et al 1983; Smolen et al 1981; Sullivan et al 1984; Brunelleschi et al, unpublished observations); PMA, which was suggested to mimic the effects of diacylglycerol, stimulated $O_2^{\cdot-}$ production without inducing a rise in cytosolic calcium (Nishizuka 1984; Westwick & Poll 1986). Rhein was suggested to possess potential chelating properties for calcium (Kean 1968). In our experiments, the drug showed inhibitory effects on FMLP-stimulated neutrophils, even when the cells were exposed to EGTA in a calcium-free medium.

The ability of rhein to interfere with $O_2^{\cdot-}$ production from human neutrophils could depend, at least partially, on its inhibitory effects on oxidase systems, since the drug was reported to inhibit competitively NADH-dehydrogenase, in-vitro (Kean et al 1971). Neutrophils require a NAD(P)H-dependent membrane oxidase for generating $O_2^{\cdot-}$ (Babior 1984).

Rhein slightly inhibited lysosomal enzyme release from FMLP-activated neutrophils and, at the highest concentrations, directly affected the activities of the enzymes that were released. These data have to be evaluated along with those obtained by Raimondi et al (1982). The capacity of some protease inhibitors (e.g., L-1-tosylamide-2-phenylethylchloromethylketone: TPCK; aprotinin) to inhibit oxy-radical production from human neutrophils was reported (Kitagawa et al 1980; Curnutte et al 1984; Hallett et al 1985).

In conclusion, rhein appeared to affect respiratory burst from human neutrophils and, to a lesser extent, lysosomal enzyme release. These inhibitory effects might account for some of the in-vivo pharmacological activities of its precursor, diacetylrhein.

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