Agents and Actions, vol. 18, 5/6 (1986)

0065-4299/86/060555-05$2.50

In vitro interactions between endogenous polyamines and superoxide anion

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Abstract

Endogenous polyamines with anti-inflammatory activity scavenge superoxide and possibly other oxy-radicals produced by xanthine oxidase or from stimulated polymorphonuclear leucocytes. Polyamines incubated with stimulated cells are in part metabolised. Putrescine is converted to metabolites tentatively identified as γ-aminobutyraldehyde, Δ'-pyrolline and γ-aminobutyric acid. The metabolism of spermidine, spermine and cadaverine was not as extensively studied but metabolites were formed that gave positive reaction to Schiffs reagent on tlc plates. The possible relevance of the results to the anti-inflammatory action of polyamines is discussed.

Introduction

In a search for endogenous compounds with anti-inflammatory activity it was found that putrescine, spermidine and spermine accumulated in inflammatory exudates [1]. Subsequently putrescine and spermidine were found to be anti-inflammatory when tested against carrageenan induced inflammation in the rat and putrescine was also anti-inflammatory against adjuvant induced arthritis in the rat [2]. The anti-inflammatory action of polyamines was also reported by OYANAGUI [3] who found that putrescine, spermidine and spermine inhibited serotonin induced paw oedema in mice and carrageenan paw oedema in rats.

The mode of action of polyamines is not known, but OYANAGUI [3] suggested that they may promote the synthesis of a vascular permeability inhibitory protein 'vasoregulin' which would have anti-inflammatory action. Electron spin resonance studies have shown that spermine has a scavenging action on superoxide anion [4]. Superoxide is produced from stimulated neutrophils and other phagocytes during inflammation. Superoxide induces the formation of other active oxygen species and is itself a powerful chemotactic agent [5]. Since polyamines accumulate in inflammatory exudates it is possible that these substances may modulate superoxide action by scavenging this anion. The present investigation was designed to establish whether polyamines have a scavenging action on superoxide produced both by the hypoxanthine/xanthine oxidase reaction and by stimulated polymorponuclear leucocytes (PMNLs). Since it is possible that PMNL may modify putrescine we also investigated the pattern of metabolites produced when putrescine was incubated with stimulated PMNLs.

Materials

Radioactive compounds were purchased from Amersham International plc, England. Tissue culture media were purchased from Flow Laboratories, Ayrshire, Scotland and other chemicals from the Sigma Chemical Co., Poole, Dorset, England unless otherwise stated. Putrescine, spermidine, spermine and cadaverine were purchased and used as the hydrochloride salts. In this paper, for comparative purposes, ethylenediaminetetraacetic acid (EDTA) is regarded as a polyamine.

Methods

The action of polyamines on superoxide production by the hypoxanthine/xanthine oxidase reaction

A solution of hypoxanthine 0.5 m*M* in phosphate buffer was prepared (0.1 *M*, pH 7.4) incorporating 50 n moles of cytochrome C(cyt C). Superoxide was generated by the addition of xanthine oxidase (0.8 units) (one unit will convert 1 μ mole of xanthine to uric acid per min at pH 7.5 at 25°C) and the reduction of cyt C followed at 550 nm (6) in a spectrophotometer (Pye-Unicam SP 500) over 10 min at 37°C. Polyamines were added at various concentrations to the reaction solutions but were omitted from the controls. In some reactions cyt C was omitted but the absorbance was followed by 295 n*M*. This measured the formation of uric acid and this reaction was followed in order to determine

whether polyamines had any direct effect on uric acid production. In addition to the reduction of cyt C the reaction was followed by the inhibition by polyamines of the photoreduction of nitroblue-tetrazolium (NBT) [7]. In this experiment the reaction mixture consisted of 5×10^{-5} *M* NBT, 10^{-2} *M* methionine, 1.17×10^{-6} *M* riboflavin, 2×10^{-5} *M* sodium cyanide, in phosphate buffer (0.02 *M*) pH 7.8. Polyamines were added over a range of concentrations. The reaction mixtures were incubated at 30°C and illuminated over 6 min by a Shandon-Southern photopol lamp. The absorbance was measured at 560 nm in a Pye-Unicam SP500 spectrophotometer. Polyamines were omitted from the controls and the results plotted as % of control values.

The action of polyamines on superoxide generated from stimulated guinea-pig or rat PMNLs

PMNLs were obtained by peritoneal lavage from male Wistar strain rats (200–250 g body weight) or male Dunkin Hartley strain guinea-pigs (400–500 g body weight) 4 h after injection of 1% aqueous oyster glycogen solution (10 ml rats; 20 ml guinea pigs). The lavage solution was heparinized ice-cold buffered culture medium EMEM supplemented with 2 m*M* glutamine and 1% v/v heat inactivated foetal calf serum (pH 7.4) using 30 ml for rats and 50 ml for guinea-pigs. After washing and viability testing by trypan blue exclusion the cells were incubated in the supplemented culture medium for 5 min at 37°C prior to use. Where polyamine-superoxide interactions were followed by cyt C reduction 10^6 guinea-pig cells were suspended in EMEM at 37°C which also contained polyamines at various concentrations and 50 n moles of cyt C. The reaction was started by the addition of phorbol myristate acetate to a final concentration of 10^{-8} M and the reaction followed at 560 n*M*. Polyamines were omitted from the controls. The procedure for measuring putrescine-superoxide interactions by chemiluminescence from rat cells consisted of dissolving putrescine at various concentrations in EMEM which also contained luminol at 2×10^{-4} *M*. Zymosan (100 μl) was added to 300 μl portions of the above mixture in a cuvette placed in a LKB-Wallace Model 125 luminometer coupled to a potentiometric recorder. The reaction was started by the addition of 5×10^5 cells. With guinea-pigs zymosan was replaced by the soluble stimulating reagent phorboal myristate acetate at 10^{-8} *M*. Putrescine was omitted from the controls. The reaction was followed for 30 min and the cells tested for viability using trypan blue exclusion. Superoxide dismutase (SOD) was used as a reference compound to suppress superoxide output. One unit of SOD will inhibit the reduction of cyt c by 50% per min at pH 7.8 at 25°C [8]. In some experiments with guinea-pig cells the reaction mixtures were supplemented with [14C]-putrescine (8.3 μCi) as a tracer and after 30 min the cells were lysed by freezing and thawing and centrifuged at 10,000 g for 30 min at 4°C. The supernatants were spotted on to silica tlc plates (20 × 5 cm) and developed in n-butanol, acetic acid, water (77:17:6 v/v); n-butanol, water, acetic acid, pyridine (4, 2, 1, 1 v/v); n-propanol, water, hydrochloric acid (60, 20, 20 v/v); n-propanol, water, triethylamine (85, 15, 3 v/v).

The plates were dried with a hair dryer and then scanned for radioactivity in a windowless gas-flow Tracerlab radiochromatogram scanner (Tracerlab). Two standards were also spotted on to the plates, [14C]-putrescine and [14C]-γ aminobutyric acid. In a parallel experiment diamine oxidase (25 units) was substituted for the cells. After scanning the plates were sprayed with Schiffs reagent to detect aldehydes and with 2% w/v ninhydrin in acetone to detect polyamines.

Results

The effect of polyamines on superoxide output from the xanthine/hypoxanthine reaction

The results are plotted in Figure 1 and show the effects of six polyamines, putrescine, spermidine, spermine, cadaverine, EDTA and diaminopropane on superoxide output as measured by the reduction of cyt C. All the polyamines inhibited the superoxide induced reduction of cyt C. The order of reactivity was: putrescine > diaminopropane > spermidine > EDTA > spermine > cadaverine. In control experiments the polyamines did not affect the formation of uric acid over the concentration range used in the scavenging experiments. Therefore polyamines had no direct effect on xanthine oxidase activity. In experiments where NBT was substituted for cyt C an identical result was obtained (Figure 2).

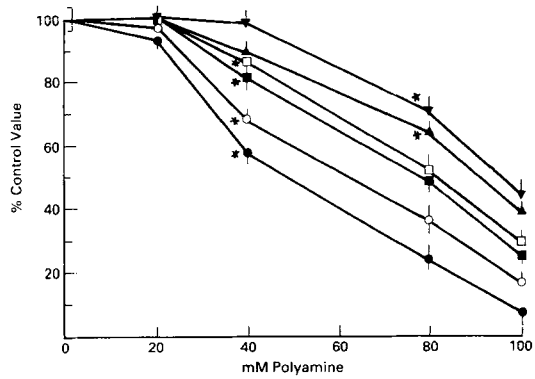

Figure 1
The effect of polyamines on the reduction of cyt.C by the products of the hypoxanthine/xanthine reaction. The results are expressed as a percentage of the control values ± SEM (4 determinations) when polyamine was omitted. The actual control value was 27.1 ± 2.3 n moles cyt.C reduced per tube (2.5 ml)/10 min. Significance was taken at $p < 0.05$ at the lowest effective drug concentration. The student t test was used throughout the experiments ●, putrescine; ○, 1,3-diaminopropane; ■, spermidine; □, EDTA; ▲, spermine and ▼, cadaverine.

The effect of polyamines on superoxide output from stimulated PMNLs on cyt C reduction

The effect of polyamines on superoxide produced from stimulated guinea-pig PMNLs is shown in Figure 3. The results are very similar

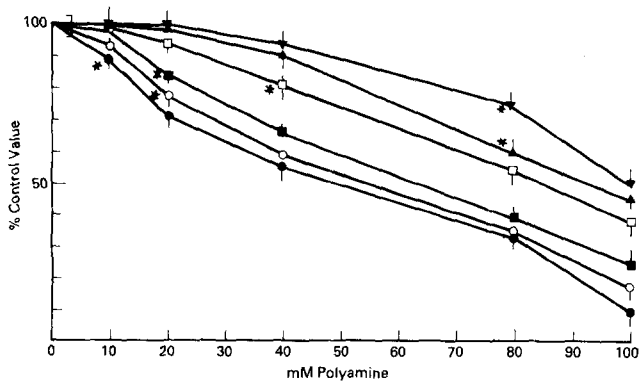

Figure 2
The effect of polyamines on the aerobic photoreduction of NBT in the presence of riboflavin. The results are expressed as a percentage of the control values when polyamine was omitted. The actual readings were absorbance values for four determinations ± SEM. Significance was taken at $p < 0.05$ at the lowest effective drug concentration. ●, putrescine; ○, 1,3-diaminopropane; ■, spermidine; □, EDTA; ▲, spermine and ▼, cadaverine.

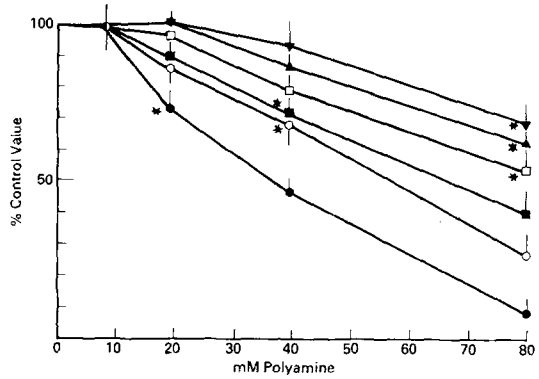

Figure 3
The effect of polyamines on cyt.C reduction in the presence of guinea-pig PMNLs stimulated with PMA. The results were recorded as percentages of control values where polyamine was omitted. The values plotted are the mean ± SEM of four determinations. The actual control value was 20.0 ± 3.2 n moles cyt.C reduced/10 cells/15 min. Significance was taken as $p < 0.05$ at the lowest effective drug concentration. ●, putrescine; ○, 1,3-diaminopropane; ■, spermidine; □, EDTA; ▲, spermine and ▼, cadaverine.

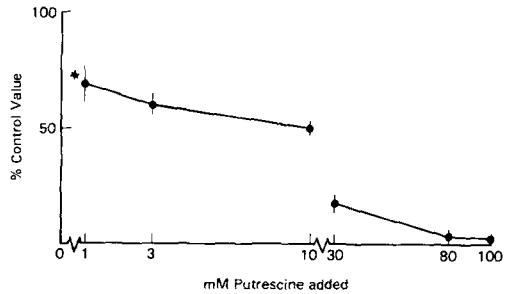

Figure 4
The effect of putrescine on chemiluminescence in the presence of rat PMNLs stimulated with zymosan. The results were recorded as readings of light intensity recorded in mV. The values plotted as percentages of controls where polyamine was omitted ± SEM for 4 determinations. Significance is recorded at $p < 0.05$ for the lowest effective drug concentration.

to those obtained when superoxide was generated from the hypoxanthine/xanthine oxidase reaction with the same order of effectiveness. A similar result was obtained when chemiluminescence was used to follow the interaction.

Chemiluminescence

The effect of putrescine on rat cells using chemiluminescence is shown in Figure 4 and that of six polyamines in Figure 5. Both figures show similar results to those obtained by cyt C reduction but the method was more sensitive. The rat cells gave similar results to guinea-pig cells for putrescine. Evidence that the chemiluminescence was produced by superoxide was obtained when superoxide dismutase was substituted for putre-

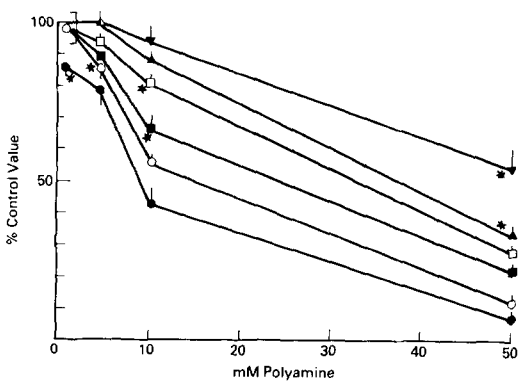

Figure 5
The effect of polyamines on chemiluminescence in the presence of guinea-pig PMNLs stimulated with PMA. The results were recorded as readings of light intensity recorded in mV. The values plotted as percentages of controls where polyamine was omitted ± SEM for 4 determinations. ●, putrescine; ○, 1,3-diaminopropane; ■, spermidine, □, EDTA; ▲, spermine and ▼, cadaverine. Significance is recorded at $p < 0.05$ for the lowest effective drug concentration.

scine in the experiments. A dose-responsive suppression was found with values $55 \pm 1.6\%$ (SOD 175 units), $37 \pm 2.3\%$ (SOD 350 units), $22 \pm 5.4\%$ (SOD 700 units) and complete suppression at concentrations in excess of 2000 units. Superoxide dismutase had no effect on urate formation. Putrescine was found to be the most effective scavenger of superoxide anion. The effectiveness of the scavenging action of the polyamines was in the same order for guinea-pig PMNLs as when superoxide was generated by the xanthine oxidase-hypoxanthine interaction. Since the polyamines interacted with superoxide generated either by xanthine oxidase or by stimulated cells it suggests that the action of polyamines was of a scavenging nature.

Metabolism of polyamines by stimulated guinea-pig PMNLs

Putrescine was found to be metabolised to metabolites tentatively identified as γ-aminobutyraldehyde ($5.6 \pm 1.6\%$ of total counts on the tlc plate), γ-aminobutyric acid ($8 \pm 4.3\%$), Δ′-pyrolline (20.7 ± 2.0) and unchanged putrescine ($65.7 \pm 3.2\%$). γ-Aminobutyraldehyde is known to be unstable and rearranges non-enzymically to the more stable product Δ′-pyrolline. Putrescine and γ-aminobutyric acid were identified by the use of their respective 14[C] standards. γ-Aminobutyraldehyde and Δ′-pyrolline were tentatively identified from their chromatographic behaviour which was compared with that of the products of the reaction where putrescine was incubated with diamine oxidase. In this experiment, the metabolite γ-aminobutyradlehyde ($29.9 \pm 1.1\%$) and Δ′-pyrolline ($15.2 \pm 4.0\%$) were detected and determined by scanning on tlc plates with residual unchanged putrescine ($54.9 \pm 2.7\%$). The spots identified as aldehyde derivatives also gave a positive reaction with Schiffs reagent. Aldehyde derivatives were also detected as major metabolites in the supernatants where spermidine or spermine was incubated with stimulated PMNLs. These were detected by spraying tlc plates with Schiffs reagent and scanning the plates for radioactive substances. The amount of Schiff positive readioactive material present was $29 \pm 5.6\%$ where spermidine was the substrate and $15 \pm 5.1\%$ with spermine. Other minor metabolites were not investigated and the bulk of spermine and spermidine appeared to be recovered unchanged.

Discussion

All six amines studied scavenged superoxide anion in an order of effectiveness: putrescine > diaminopropane > spermidine > EDTA > spermine > cadaverine. All the amines are primary amines but spermidine and spermine also possess secondary amine groups. Electron spin resonance (ESR) data show reaction between superoxide and spermine [4] and it would be of interest for a comparative ESR study of the six amines to be made.

In our previous work we found that putrescine and spermidine were anti-inflammatory against carrageenan induced oedema in the rat [2]. Of the other polyamines, spermine has been reported to have anti-inflammatory action [3] although we did not find this against carageenan oedema in our laboratory. It is possible that this was due to different strains of rats being used for the experiments. Diaminopropane is also anti-inflammatory against carrageenan induced inflammation (unpublished results). EDTA inhibits lipid peroxidation [9]. Cadaverine was not found to have anti-inflammatory activity against carrageenan induced oedema either in our laboratory or elsewhere [3]. Whether the scavenging activity of polyamines has any significant anti-inflammatory effect *in vivo* requires further investigation. The plasma levels of endogenous polyamines are very low – normal human serum [10] gives values for putrescine as 155 ± 122 spermine, 17 ± 16, and spermidine 45 ± 35 n moles/L. Clearly these values are far lower than the ones used in our experiments. However, human granulocyte levels are far higher [11] putrescine $468 \pm 244/10^9$ cells, spermine $548 \pm 183/10^9$ cells and spermidine 241 ± 156 n moles/10^9 cells. An additional factor that may be relevant is that during inflammation polyamine levels are increased in inflammatory exudates [1, 2] and it is known that stimulated macrophages and T-lymphocytes show sharply induced ornithine decarboxylase activity [12, 13]. Oxygen-induced lung injury in rats significantly increases ODC, putrescine, spermidine and spermine levels and inhibition of this elevation by DFMO adversely affects repair [14]. If polyamines do scavenge superoxide *in vivo* it is likely that this effect is a local one either within the cell or in the immediate environment surrounding the cell and that the effect will be localised to sites of inflammatory foci. Such a

site in rheumatoid arthritis may be the cartilage-pannus junction. Putrescine was metabolized by stimulated polymorphonuclear leucocytes to γ-aminobutyraldehyde, γ-aminobutyric acid and Δ′ pyrolline. The formation of γ-aminobutyric acid from putrescine has been reported in rat liver and brain [15] and in mice [16] where diamine oxidase was shown to be involved in the pathway. Both spermine and spermidine were also converted to aldehyde derivatives by stimulated cells although the metabolites were not identified. Amino-aldehyde derivatives of polyamines are extremely unstable compounds and it is possible that these metabolites may scavenge active-oxygen species produced by stimulated phagocytes. This might explain why putrescine which as ten times more anti-inflammatory than spermidine in inhibiting carrageenan induced oedema in the rat [1] but was only just over twice as effective as spermidine in scavenging superoxide. Clearly the properties of these metabolites need further investigation.

Although polyamines scavenge superoxide it is possible that they may also scavenge other oxy-species produced from superoxide such as hydrogen peroxide which gives rise to the damaging hydroxyl radical. A likely role for polyamines *in vivo* is that the rise in the levels of these compounds associated with inflammation contribute to an endogenous anti-oxidant pool of compounds such as ascorbic acid, thiol compounds and tocopherols [4] with scavenging properties against superoxide and its associated oxy-species. This effect would be anti-inflammatory. A suggestion that polyamines may act as cellular anti-oxidants was made previously [4].

Acknowledgments

We acknowledge financial assistance from the Oliver Bird fund for rheumatism, the Arthritis and Rheumatism Research Council and the Egyptian Government. AMLK is the holder of a British Government ORS scholarship. The Wellcome Trust is thanked for a grant to purchase the Radiochromatogram scanner and the Cystic Fibrosis Trust for Funds for the purchase of a LKB-Wallac 1250 chemiluminometer.

Received 18 October 1985; accepted 12 March 1986

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