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STIMULATORY EFFECT OF PLATINUM(IV) ION ON THE PRODUCTION OF SUPEROXIDE RADICAL FROM XANTHINE OXIDASE AND MACROPHAGES

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Abstract—Superoxide radical ($\cdot O_2^-$) production was measured spectrophotometrically using NADH and lactate dehydrogenase (LDH) in a xanthine oxidase(XOD) plus hypoxanthine (HX) system and in an isolated guinea pig macrophages system. Sodium platinum (IV) chloride (Na_2PtCl_6: 2.5×10^{-4}–1×10^{-3} M) enhanced the production of $\cdot O_2^-$ in both systems (2–10 times). The degree of the enhancement was dependent on incubation time, basal level of $\cdot O_2^-$ production and concentration of Na_2PtCl_6. The stimulated $\cdot O_2^-$ production in the XOD system was inhibited by luminol (*O*-aminophtalhydrazide) and that in the macrophages was inhibited by an anti-inflammatory drug, Diclofenac sodium (Dc). These results show that platinum (IV) ion is either a potent stabilizer of $\cdot O_2^-$ or a stimulator of $\cdot O_2^-$ production as are paraquat or streptonigrin. This specific character of platinum (IV) ion may explain its bactericidal and inflammation-inducing properties.

Many metal ions such as Mn^{+2}, Hg^{+2}, Cu^{+2}, Fe^{+2}, Fe^{+3}, Ni^{+2}, Co^{+2} in concentrations of 10^{-6}–10^{-3} M inhibit the production of superoxide radical ($\cdot O_2^-$) from isolated guinea-pig macrophages [1]. Other metal ions such as Ca^{+2}, Ba^{+2}, Cd^{+2} and Pb^{+2} inhibit $\cdot O_2^-$ production to a far less extent at a concentration of 10^{-3} M. Pt^{+4} (Na_2PtCl_6) even stimulates $\cdot O_2^-$ production. $\cdot O_2^-$ from granulocytes is known to be bactericidal [2, 3], and certain platinum compounds have been reported to inhibit cell division of gram-negative bacteria [4]. Excess production of $\cdot O_2^-$ may be cause of inflammation. The prostaglandin phase of rat carrageenan foot oedema is completely inhibited by intravenous injections of superoxide dismutase (SOD: 0.5–2.0 mg/kg) which specifically breaks down $\cdot O_2^-$ [5]. Platinum complexes are also reported to induce human atopic hypersensitivity [6]. Many *in vivo* effects of the platinum compounds seem to be explained by the ability of Pt^{+4} ions to stimulate $\cdot O_2^-$ production.

MATERIALS AND METHODS

The absorbance at 340 nm of NADH (0.96 μmole) was continuously recorded with a Shimazu Multipurpose MPS-5000 spectrophotometer at 37°. Superoxide dismutase (SOD: Sigma's product from bovine blood), which is known to react specifically with $\cdot O_2^-$, completely inhibited the NADH oxidation induced by 0.1 U/ml xanthine oxidase (XOD: from buttermilk) plus 80 μM hypoxanthine (HX) or by macrophage suspensions. Reaction mixtures in this experiment contained 5 μM lactate dehydrogenase (LDH: from rabbit muscle) and either 8 mM Veronal–acetate–HCl buffer pH 6.5 or 125 mM NaH_2–Na_2HPO_4 buffer pH 6.5. In some experiments, 25 mM phosphate buffer was also used. Phosphate anion is essential for the production of $\cdot O_2^-$ from macrophages and the optimum pH for its production is 6.5. The details were reported in the preceding report [1]. The method used in the present experiment to determine the amount of $\cdot O_2^-$ is a modification of that of Chan *et al.* [7], who introduced the LDH and NADH method.

Small amounts of $\cdot O_2^-$ were produced even in media containing no XOD or macrophages, and this production was influenced by various agents. Stimulatory effects of agents on $\cdot O_2^-$ production were, therefore, calculated by the following formula:

$$\text{Stimulation (\%)} = \frac{\Delta A(\text{agent} + \text{S}) - \Delta A(\text{agent only})}{\Delta A(\text{S only}) - \Delta A(\text{medium only})}$$

where ΔA is the difference in absorption at 340 nm before and after the reaction, and S is the source of $\cdot O_2^-$ i.e. XOD + HX or macrophage suspension. The pH of all the agents used was adjusted to 6.5 before addition. Diclofenac sodium (Dc,) and luminol solutions contained up to 0.1% of *N*,*N*′-dimethylformamide (DMF) to effect solution: this amount had little effect on $\cdot O_2^-$ production.

RESULTS

The degree of NADH oxidation was linear up to 10 min with 0.1 U/ml XOD plus 80 μM HX (Fig. 1). This oxidation was more than doubled by addition of Na_2PtCl_6 (5×10^{-4} M) and was nearly completely inhibited with SOD (14 U/ml = 4 μg/ml). Without XOD, Na_2PtCl_6 had no stimulatory effect on basal $\cdot O_2^-$ production. At higher Na_2PtCl_6 concentrations, the interval of linear oxidation was shortened. Moreover, at 2.5×10^{-3} M the platinum became inhibitory at 5 min; a lower Na_2PtCl_6 concentration of 2.5×10^{-4} M required a time lag of at least 5 min to stimulate $\cdot O_2^-$ production.

The stimulation of NADH oxidation was not due to the stimulation of LDH activity by Na_2PtCl_6. The LDH activity was measured in terms of the decrease

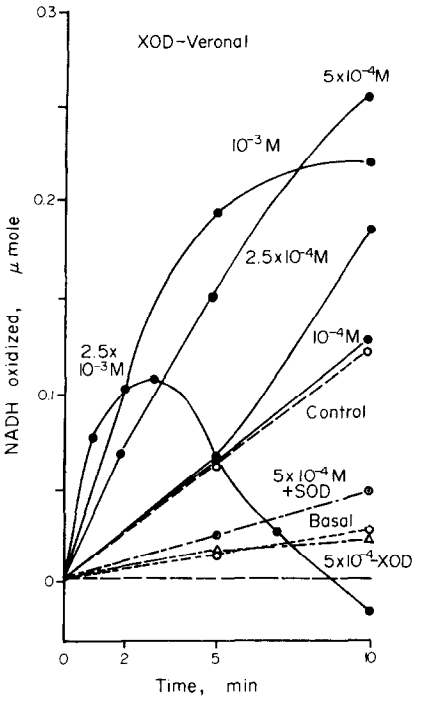

Fig. 1. Time-course of $^{\cdot}O_2^-$ production effected by 0.1 U/ml XOD plus 80 μM HX in various concentrations of Na_2PtCl_6 in 8 mM Veronal–acetate–HCl buffer pH 6.5. The final concentration of SOD added was 14.0 U/ml (= 4 μg protein/ml).

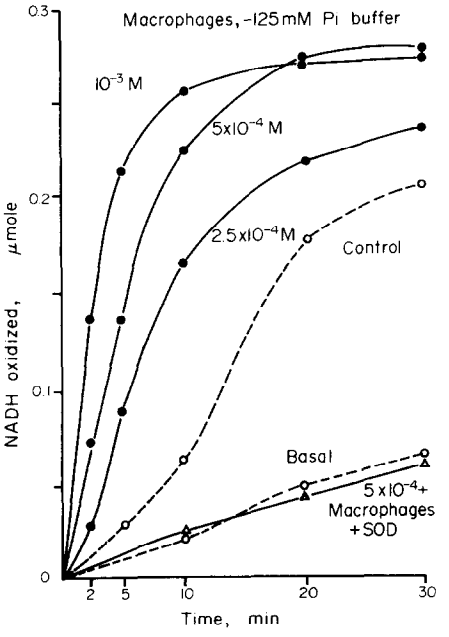

Fig. 2. Time-course of $^{\cdot}O_2^-$ production effected by 2.6×10^6 macrophages/ml in various concentrations of Na_2PtCl_6 in 125 mM NaH_2–Na_2HPO_4 buffer pH 6.5. The final concentration of SOD added was 14.0 U/ml.

in NADH absorption at 340 nm in 30 sec incubation with 0.1 mM sodium pyruvate and 8×10^{-3}U/ml LDH at 37°. Na_2PtCl_6(10^{-4} M–10^{-3} M) never stimulated the LDH activities. In a concurrently conducted experiment, sodium oxamate (a specific LDH inhibitor) inhibited the LDH activity by 94 per cent at a concentration of 10^{-3} M and 74 percent at 10^{-4} M.

The $^{\cdot}O_2^-$ production by the isolated guinea-pig macrophages was also stimulated by Na_2PtCl_6 (Fig. 2). These findings suggest that the platinum salt stabilizes $^{\cdot}O_2^-$ or facilitates $^{\cdot}O_2^-$ production. The platinum salt may activate the XOD system or enzyme(s) that

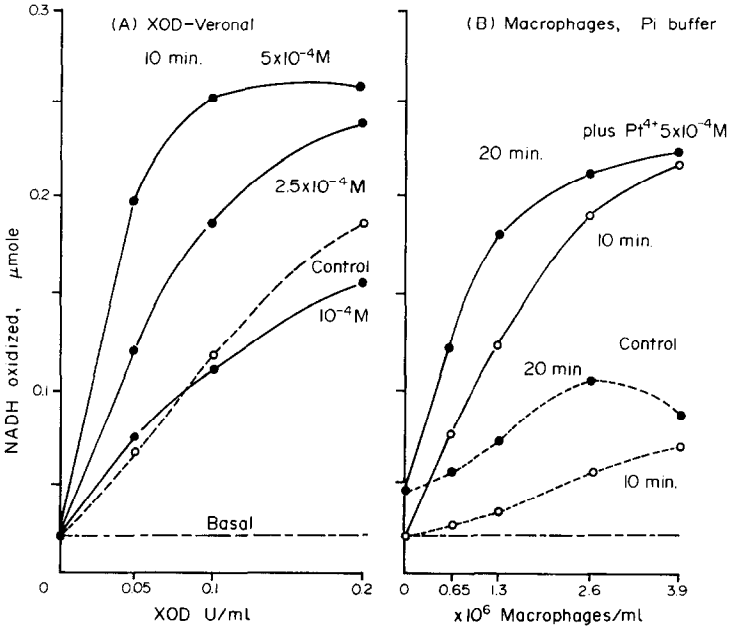

Fig. 3. Stimulation of $^{\cdot}O_2^-$ production by Na_2PtCl_6 in different $^{\cdot}O_2^-$ producing conditions. Reaction mixture contains buffer solutions, LDH, macrophage suspension or XOD + HX and Pt^{+4}(—) or no Pt^{+4}(– – –) solutions. Reaction was started by the addition of NADH and incubated for 10 or 20 min. (A) 0.1 U/ml XOD + 80 μM HX in 8 mM Veronal–acetate–HCl buffer pH 6.5. (B) Macrophages in 125 mM NaH_2–Na_2HPO_4 buffer pH 6.5.

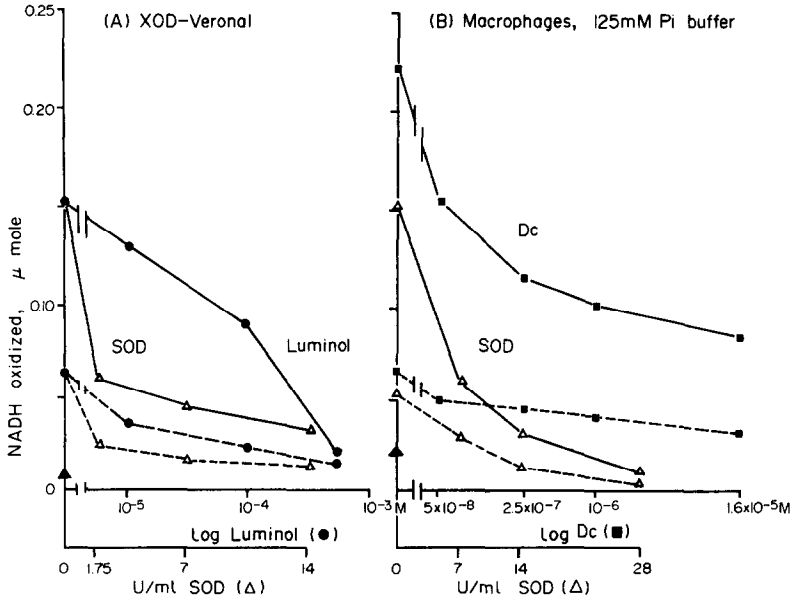

Fig. 4. Inhibition of control and Pt^{+4}-stimulated $^{\cdot}O_2^-$ productions by SOD (△), luminol (●) and Diclofenac sodium (Dc : ■). Control (---) and with 5×10^{-4} M Na_2PtCl_6 (—). (A) 0.1 U/ml XOD + 80 μM HX in 8 mM Veronal–acetate–HCl buffer pH 6.5 was incubated for 5 min. (B) 1.8×10^6 macrophages/ml for SOD test or 2.6×10^6 macrophages/ml for Dc test in 125 mM NaH_2–Na_2HPO_4 buffer pH 6.5 was incubated for 10 min. (▲) NADH oxidation by 5×10^{-4} M Na_2PtCl_6 without XOD + HX or macrophages.

produce $^{\cdot}O_2^-$ in macrophages, but the exact mechanism of enhanced $^{\cdot}O_2^-$ production by Pt^{+4} is not clear. Fig. 3 shows the differences in $^{\cdot}O_2^-$ stimulation under various $^{\cdot}O_2^-$ producing conditions. In both the XOD and macrophage systems, the stimulation by Na_2PtCl_6 is greater in conditions where small amounts of $^{\cdot}O_2^-$ are being produced. Factors that govern the stimulatory effect comprise incubation time, concentration of Na_2PtCl_6 and the amount of $^{\cdot}O_2^-$ production. Phosphate anion concentration is important in the case of macrophages [1].

Fig. 4 shows that the stimulation of NADH oxidation by Na_2PtCl_6 is due to $^{\cdot}O_2^-$ production and not to other oxidants, because SOD inhibited the Pt^{+4}-stimulated NADH oxidation. The inhibition was dose-dependent just as in the control NADH oxidation by the XOD system and by the macrophage suspension. Luminol, a $^{\cdot}O_2^-$ scavenger [8], inhibited NADH oxidation in the XOD system. Diclofenac sodium (Dc), a non-steroidal anti-inflammatory drug and a potent inhibitor of $^{\cdot}O_2^-$ production in the macrophage system (not in the XOD system), demon-

Table 1. Stimulation of $^{\cdot}O_2^-$ production effected by Na_2PtCl_6

$^{\cdot}O_2^-$ generating source	Buffer	Time (min)	Control net NADH oxidation (μmole)	Stimulation ratio (Na_2PtCl_6 conc. M) 2.5×10^{-4}	5×10^{-4}	1×10^{-3}
XOD (U/ml)						
0.05	V	5	0.020	7.2	10.7	—
0.10	V	5	0.058	1.1	2.1	2.4
0.20	V	5	0.092	2.0	2.4	—
0.05	P(b)	10	0.078	1.0	1.9	3.0
Macrophages ($\times 10^6$/ml)						
2.6	P(a)	10	0.011	4.0	6.6	9.3
2.6	P(a)	20	0.045	3.0	3.2	3.8
2.6	P(b)	5	0.026	3.7	4.8	7.9
2.2	P(b)	5	0.018	3.2	6.6	—
2.6	P(b)	10	0.041	3.6	5.1	—
2.8	P(b)	10	0.042	2.8	5.1	—
2.2	P(b)	10	0.034	2.4	5.4	—

The stimulatory ratio was calculated as described in the text. Control net NADH oxidation was set as 1.0. V, 8 mM Veronal–acetate–HCl buffer pH 6.5; P(a), 25 mM NaH_2–Na_2HPO_4 buffer pH 6.5; P(b), 125 mM NaH_2–Na_2HPO_4 buffer pH 6.5.

strated dose-dependent inhibition of the NADH oxidation which was stimulated by 5×10^{-4} M Na_2PtCl_6. Pt^{+4} can perhaps be applied for screening anti-inflammatory drugs with economy of macrophages [1].

DISCUSSION

To my knowledge only a small number of agents have been reported to stimulate $^{\cdot}O_2^-$ production. Curnutte *et al.* [9] showed that fluoride (20 mM) increased $^{\cdot}O_2^-$ production about 5 times at 10 min and 2 times at 20 min when measured by the absorbance of cytochrome *c* reduced by human granulocytes. This stimulation was verified in our study using guinea-pig macrophages and a LDH + NADH assay system, but was not as marked as in their study, and disappeared after 20 min Cytochalasin B (5 μg/ml), contrary to the results of Curnutte's study, was shown to inhibit $^{\cdot}O_2^-$ production by macrophages [1]. This discrepancy may be due to differences in the assay method. Cytochrome *c* might have the wrong configuration to allow this enzyme to trap quickly $^{\cdot}O_2^-$ generated on the cell membrane [10, 11]. Cytochalasin E was stimulatory when examined by the nitroblue tetrazolium assay and guinea-pig leucocytes [10]. Paraquat (a herbicide) damaged human lungs [12, 13] and was reported to enhance $^{\cdot}O_2^-$ production *in vitro* [14]. Streptonigrin (an antibiotic, 1.0 μg/ml) was non-bactericidal but when combined with a small amount of $^{\cdot}O_2^-$, regenerated enough $^{\cdot}O_2^-$ to become bactericidal [15].

Na_2PtCl_6 seems to fall in the category of these kinds of stimulators of $^{\cdot}O_2^-$ production or to be an efficient $^{\cdot}O_2^-$ stabilizer. This platinum salt is easily available from commercial sources, and is hopefully applicable for detecting traces of $^{\cdot}O_2^-$ generated from various materials. Furthermore, the platinum salt makes possible the use of smaller amounts of macrophages for screening anti-inflammatory agents [1].

The inflammation-inducing capacity of Na_2PtCl_6, was also studied (unpublished result). A saline solution of Na_2PtCl_6 (4 μmoles/guinea-pig paw) caused acute lethal effect, but when injected as 10% water in 90% vegetable oil suspension, it caused chronic paw swelling and profound damage of joint connective tissues and of bones from 3 days to more than 2 weeks. Equimolar amounts of copper acetate, ferric chloride, lead chloride and cadmium chloride caused no such swelling or damage.

$(NH_3)_2PtCl_4$, $(NH_3)_2PtCl_6$ and their derivatives, which are known as anti-tumor agents [16], were not available so their effects on $^{\cdot}O_2^-$ production could not be studied. These complex salts are reported to destabilize the DNA-helix *in vitro* [17]. Other anti-tumor agents, Bleomycin [18] and Mitomycin C [19] are also supposed to attack tumor cells by the formation of $^{\cdot}O_2^-$. There is no report that Na_2PtCl_6 itself has an anti-tumor effect, but it is worth considering the relationship between the $^{\cdot}O_2^-$ production and the anti-tumor effect of platinum compounds. $(NH_3)_2PtCl_4$ is reported to accumulate in plasma and in the organs [20].

Platinum compounds also depress the skin allograft reaction in mice [21] and inhibit production of antibody to sheep erythrocytes in mice spleen cells [22]. The stimulatory effect of Pt^{+4} ions on $^{\cdot}O_2^-$ production may explain the effect observed in *in vitro* studies.

REFERENCES

1. Y. Ōyanagui, *Biochem. Pharmac.* **25,** 1473 (1976).
2. R. L. Baehner and D. G. Nathan, *New Engl. J. Med.* **278,** 971 (1968).
3. J. T. Curnutte and B. M. Babior, *J. clin. Invest.* **53,** 1662 (1974).
4. C. W. Welsch, *J. natn Cancer Inst.* **47,** 1071 (1971).
5. Y. Ōyanagui, *Biochem. Pharmac.* **25,** 1465 (1976).
6. A. Khan, J. M. Hill, W. Grater, E. Loeb, A. MacLellan and N. Hill, *Cancer Res.* **35,** 2766 (1975).
7. P. C. Chan and H. J. Bielski, *J. biol. Chem.* **249,** 1317 (1974).
8. L. Greenlee, I. Fridovich and P. Handler, *Biochemistry* **1,** 779 (1962).
9. J. T. Curnutte and B. M. Babior, *Blood* **45,** 851 (1975).
10. A. Nakagawara and S. Minakami, *Biochem. biophys. Res. Commun.* **64,** 760 (1975).
11. D. Roos, W. T. Homan-Müller and R. S. Weening, *Biochem. biophys. Res. Commun.* **68,** 43 (1976).
12. A. P. Autor, *Life Sci.* **14,** 1309 (1974).
13. D. S. Davies and D. L. Davies, *Fedn. Proc.* **33,** 228 (1974).
14. J. S. Bus and J. E. Gibson, *Biochem. biophys. Res. Commun.* **58,** 749 (1974).
15. E. M. Gregory and I. Fridovich, *J. Bact.* **114,** 1193 (1973).
16. B. Rosenberg and L. VanCamp, *Cancer Res.* **30,** 1799 (1970).
17. H. C. Harder, *Chem. Biol. Interactions* **10,** 27 (1975).
18. R. Ishida and T. Takahashi, *Biochem. biophys. Res. Commun.* **66,** 1432 (1975).
19. K. Handa and S. Sato, *Gann* **66,** 43 (1975).
20. C. L. Litterst, J. E. Gram, R. L. Dedrick, A. F. Leroy and A. M. Guarino, *Cancer Res.* **36,** 2340 (1976).
21. G. Brambilla and A. Maura, *Cancer Chemother. Rep.* (Part 1), **58,** 633 (1974).
22. M. C. Berenbaum, *Br. J. Cancer* **25,** 208 (1971).