METALS INHIBIT RIBOFLAVIN-CATALYZED
GENERATION OF SUPEROXIDE ANION IN
VITRO

LANCE S. TERADA, JONATHAN A. LEFF,
DAVID N. GUIDOT, GAYLE A. SHIBAO, and
JOHN E. REPINE

Department of Medicine and
Webb-Waring Lung Institute
University of Colorado Health Sciences Center
Denver, Colorado

Abstract—We found that addition of cationic metals inhibited flavin-catalyzed superoxide anion (O$_2^-$) production in vitro. Inhibition of O$_2^-$ generation by metals appeared to relate to the ability of metal ions to chelate or complex with amine groups, altering their electronegativity. Metal inhibition of O$_2^-$ production has important implications for biological systems involving O$_2^-$ radical production, as well as for assays requiring the generation of O$_2^-$ in vitro.

INTRODUCTION

Superoxide anion (O$_2^-$) is a reactive oxygen metabolite that has been implicated in a number of basic reactions in both physiologic and disease states (1, 2). The most common biologic sources of O$_2^-$ are flavin derivatives, including certain flavin-containing enzymes such as xanthine oxidase, aldehyde oxidase, and dihydroorotic dehydrogenase (3). However, O$_2^-$ is also a product of free flavin-catalyzed photooxidation of a number of amines (4, 5). This latter group of reactions uses light-activated free riboflavin to facilitate electron transfer from amine groups to O$_2$, resulting in amine oxidation and formation of reduced, highly reactive O$_2$ species. This general class of reactions is thought to have been important in evolutionary chemistry and is also relevant to certain biological systems. In addition, these reactions have been used as a source of O$_2^-$ for experiments in vitro and as the basis for a convenient assay for superoxide dismutase (SOD) (6). Superoxide anion is also produced by the flavin-indepen-
dent autooxidation of amines, and this process is catalyzed by metal ions (7, 8). Hence, we sought to determine the effect of metals on the flavin-catalyzed photooxidation of amines in vitro.

MATERIALS AND METHODS

Reagents. Riboflavin, EDTA Na₂, DL-methionine, (-)-nicotine, NaCl, phosphate salts, calcium chloride, magnesium chloride, ferric chloride, cupric sulfate, chromium chloride, manganese chloride, tungsten sodium, superoxide dismutase (SOD, bovine erythrocyte, 3000 units/mg), and cytochrome c (horse heart, type VI) were all obtained from Sigma Chemical Co. (St. Louis, Missouri). Aluminum potassium sulfate was purchased from J.T. Baker Chemical (Philipsburg, New Jersey) and catalase (bovine liver, 81,536 units/mg) was obtained from Worthington Biochemical (Freehold, New Jersey).

Generation and Measurement of O₂⁻. O₂⁻ production was measured as SOD-inhibitable reduction of cytochrome c. In a reaction volume of 1 ml, cytochrome c (1.5 mg/ml) was added to phosphate-buffered saline (20 mM, pH 7.4) containing riboflavin (1 μM), an electron-donating amine (1 mM), and the metal salt (2 mM) in the presence or absence of SOD (100 μg/ml). Catalase (10 μg/ml) was added to all tubes to prevent reoxidation of cytochrome c by H₂O₂. Samples were incubated in direct sunlight for 30 min at room temperature, wrapped in foil, and placed on ice. Absorbance at 550 nm was monitored and the rate of O₂⁻ generation calculated using an extinction coefficient (reduced-oxidized cytochrome c) of 2.10 × 10⁴/cm/M (9). In some experiments, the concentrations of the amine and the metal were varied from 0 to 2 mM.

RESULTS

Effect of Ca²⁺ on O₂⁻ Generation by Riboflavin/EDTA. The rate of production of O₂⁻ was inversely related to the concentration of added Ca²⁺ ion for any given concentration of EDTA (Figure 1). Furthermore, the inhibitory effect of Ca²⁺ was reversed by increasing the concentration of EDTA in the reaction mixture to a concentration greater than that of Ca²⁺, suggesting that inhibition may be due to chelation of Ca²⁺ by EDTA.

Effect of Various Metals on O₂⁻ Generation by Different Riboflavin/Amine Systems. Methionine and nicotine both caused O₂⁻ to be generated at a somewhat slower rate than EDTA (Table 1). However, all of these amines were inhibited by addition of cationic metals. Addition of calcium and magnesium had a much greater inhibitory effect on EDTA than on nicotine and methionine, whereas aluminum and chromium had a lesser inhibitory effect on EDTA than on nicotine and methionine. Addition of copper and manganese strongly inhibited O₂⁻ production by all three amines. By comparison, iron had a greater inhibitory effect on nicotine than on EDTA or methionine, while tungsten had only a moderate effect on both nicotine and methionine and essentially no effect.