Free Rad. Res. Comms., Vols. 12-13, pp. 499-508
Reprints available directly from the publisher
Photocopying permitted by license only

© 1991 Harwood Academic Publishers GmbH
Printed in the United Kingdom

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

HYDROGEN PEROXIDE DEPENDENT OXIDATIVE DEGRADATION OF DNA BY COPPER EPINEPHRINE

J. ARONOVITCH, D. GODINGER and G. CZAPSKI[+]

Department of Molecular Biology, School of Medicine and [+]Department of Physical Chemistry, The Hebrew University, Jerusalem 91010, Israel

The hydrogen peroxide dependent oxidation of the epinephrine-copper complex to adrenochrome is mediated by free copper ions. The oxidation is enhanced by chloride ions and by the presence of serum albumin. The reaction is not inhibited by SOD or by hydroxyl radical scavengers.

The 2:1 epinephrine or dopamine:Cu(II) complexes are able to bind to DNA and to catalyze its oxidative destruction in the presence of hydrogen peroxide. The DNA-epinephrine-Cu(II) terenary complex has characteristic spectral properties. It has the capacity to catalyze the reduction of oxygen or H_2O_2 and it preserves the capacity over a wide range of complex:DNA ratios. The rate of DNA clevage is proportional to the rate of epinephrine oxidation and the rate determining step of the reaction seems to be the reduction of free Cu(II) ions. The ability to form redox active stable DNA ternary complexes, suggests that under specific physiological conditions, when "free" copper ions are available, catecholamines may induce oxidative degradation of DNA and other biological macromolecules.

KEY WORDS: Catecholamine oxidation, copper, hydrogen peroxide, DNA complex, oxygen radicals, DNA clevage.

INTRODUCTION

The cytotoxicity of biologically active catecholamines (CA) and the side effects which they produce are thought to be related to the production of damaging free radicals and o-quinones.[1-7] Epinephrine, norepinephrine, dopamine, isoproterenol and related compounds form complexes with transition metal ions,[8-11] and may drive Fenton-like reactions in the presence of hydrogen peroxide, producing hydroxyl radicals or higher oxidation states of the metal ions.[12,13]

We found that CA-metal complexes are bactericidal in the presence of H_2O_2 and the epinephrine-copper complex kills *E. coli* cells in the absence of added H_2O_2 under anoxia.[14] Since catecholamine-copper chelates form mixed complexes with various biological macromolecules,[15] the cytotoxicity of catecholamines may be a reflection of the potential of such ternary complexes to be oxidized in the presence of H_2O_2, assuming that the formation of ternary complexes with DNA or with other macromolecules may enhance CA toxicity by induction of site directed Fenton reactions.

Oxidation of epinephrine-copper and the epinephrine-copper -DNA ternary complex by H_2O_2 were investigated spectrophotometrically at pH 6-6.5 measuring the production of adrenochrome.

RIGHTSLINK

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade. Reagents were prepared in triple distilled water and used without further purification.

Potassium phosphate, sodium chloride, cupric sulfate and hydrogen peroxide (Merck); 2[N-morpholino]ethanesulfonic acid (MES), superoxide dismutase (SOD), catalase from bovine liver 2x crystallized, epinephrine (EN), polyethylene glycol 4000(PEG) and deoxyribonucleic acid from calf thymus type I (DNA) (Sigma); albumin fraction V from bovine plasma (BSA) (Armour Pharmaceutical Co.); EDTA disodium salt, glucose and mannitol (BDH); acetic acid (Frutarom); Tris (Serva). λ-DNA was prepared as described by Maniatis.[16]

Methods

EN-copper complexes were prepared as previously described.[14] Absorbance of the $(EN)_2$-Cu(II) complex was measured at 295 nm against a reference cuvette containing all additions except copper.

Oxidation of EN by H_2O_2 was followed by measuring the absorption of the adrenochrome formed at 480 nm, using a Uvikon 860 spectrophotometer and a molar extinction coefficient of 4500. The reaction mixture contained 10 mM MES buffer pH 6.5 and the freshly prepared EN-copper complex at the desired concentration (for specifics see legends to figures). The reaction was carried out at 37° C and was initiated by the addition of H_2O_2. The final volume of the reaction mixture was 1 ml.

Electrophoretic studies were performed as previously described.[17]

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RESULTS

The EN-Cu(II) Complex

The non enzymatic oxidation of EN in the presence of O_2 is metal dependent, Cu(II) being the most active transition metal.[8] At neutral pH, EN forms primarily catecholate complexes of the $(EN)_2$-Cu(II) type.[10,11] We have studied the complex formation in MES buffer in the pH range of 5.5–6.5. The $(EN)_2$-Cu(II) complex has typical absorption bands with maxima at 240 and 295 nm. Complex formation is pH dependent i.e. upon dissociation of the EN ligand as the catecholate ion. The composition of the Cu(II) complex with EN was determined by the method of continuous variation introduced by Job. We confirmed that a 2:1 EN:Cu(II) complex is the dominating species as observed previously with other buffer systems.[10] The maximal amount of complex at pH 6 was formed when the EN/Cu(II) ratio was 1/8 (EN 0.2–0.6 mM), and the apparent molar extinction of the complex at 295 nm was 14200. Similar amounts of complex were formed in air and under anoxia. When we tried to correlate the cytotoxic effect with the rate of EN oxidation we had to use low concentrations of copper (2–4 μM) with excess EN (EN/Cu = 32/1). Under these conditions only about 60% of the copper ions were complexed.

Oxidation of $(EN)_2$-Cu(II) by Hydrogen Peroxide

The oxidation of the $(EN)_2$-Cu(II) complex to adrenochrome at pH 6–6.5, in air is

RIGHTSLINK

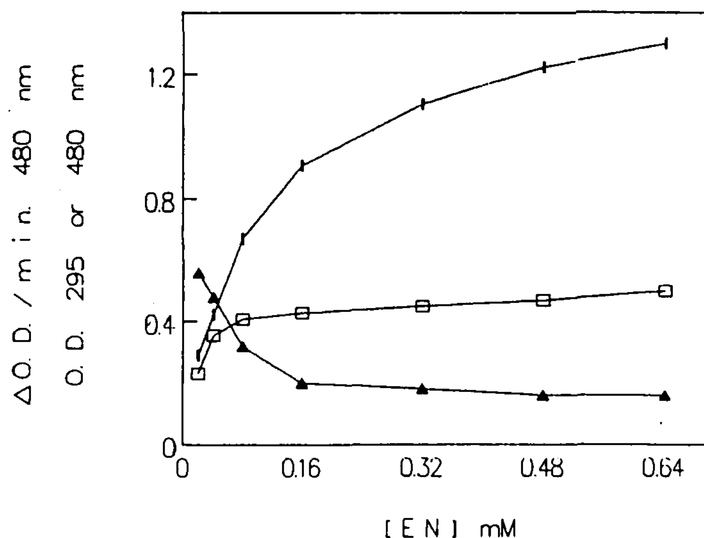

FIGURE 1. EN oxidation rate and adrenochrome production as a function of $(EN)_2$-Cu(II) concentration. Cu(II) 0.2 mM; H_2O_2 1 mM.
▲---▲ "initial" rate of oxidation, OD 480 nm/min × 40 (0-1 minute) □---□ $(EN)_2$-Cu(II) complex, OD 295 nm × 4; | --- | adrenochrome production, OD 480 nm × 3.

slow, although EN rapidly reduces copper-neocuproin to the cuprous complex. Further oxidation of the adrenochrome to melanine-like compounds is likewise slow, less than 5% of the rate with H_2O_2. Therefore, most experiments with H_2O_2 were performed in air.

Using excess H_2O_2 and constant [EN], the rate of EN oxidation to adrenochrome increases with [Cu(II)], while with constant [Cu(II)] the "initial" rate of oxidation decreases with [EN] i.e. the rate of oxidation is proportional to the concentration of free copper ions (Figure 1).

TABLE I
Rate of oxidation and adrenochrome formation. Effect of SOD, ligands and OH· scavengers.

[EN] μM	Additives	Oxidation rate OD/min. (× 10) "initial" velocity (0-1 minutes)	"maximal" velocity at (x) minutes	⁺EN oxidized to adrenochrome (%)
4	-	0.140	0.180 (2)	100
16	-	0.100	0.130 (2)	73
128	-	0.010	0.035 (5)	23
128	SOD	0.010	0.030 (5)	-
128	PEG	0.015	0.060 (6)	-
128	Mannitol	0.010	0.032 (5)	-
128	BSA	0.152	0.152 (3)	35
128	DNA	0.005	0.170 (12)	50
128	NaCl	0.060	0.280 (5)	42

Cu(II) 4μM; H_2O_2 2mM; SOD 100 μg/ml; mannitol 20 mM; PEG 1%; BSA 200 mg/ml; calf thymus DNA 100 ug/ml; NaCl 10 mM.
⁺per cent of total amount of EN oxidized to adrenochrome was calculated for the various $[EN]_0$ assuming an apparent E $(M_{-1}cm^{-1})$ of 4500.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

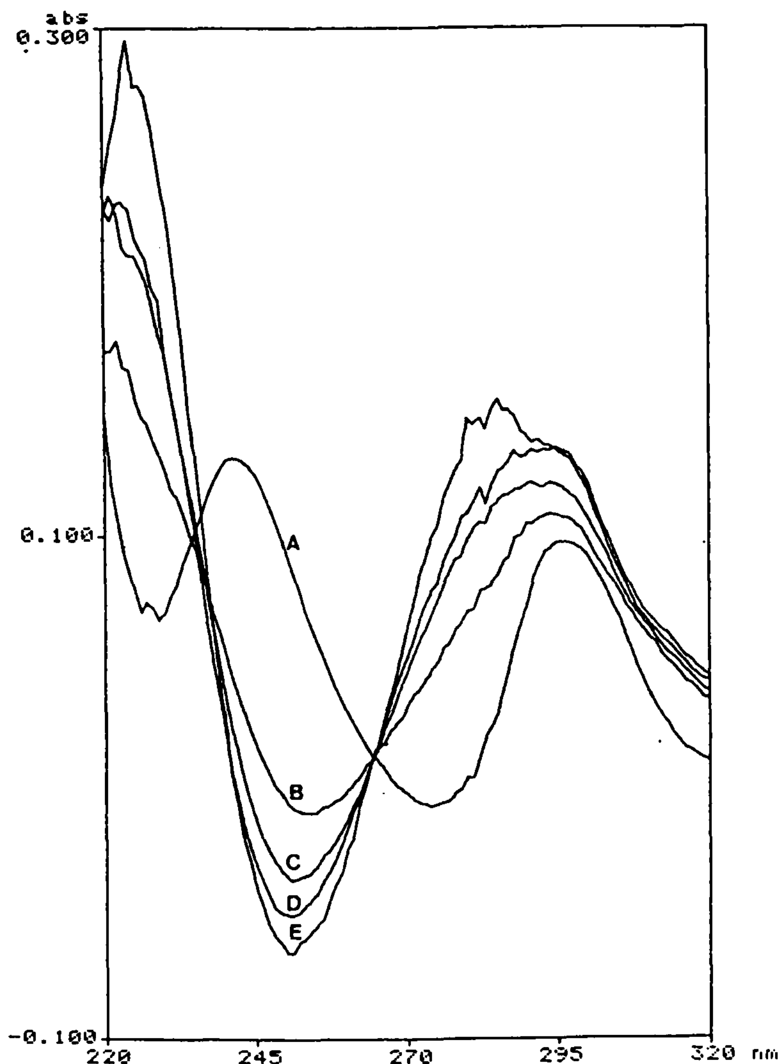

FIGURE 2. Absorption spectrum of the EN-Cu-DNA ternary complex. Blanks contained EN with the appropriate amount of DNA. EN 0.3 mM; Cu(II) 0.015 mM; calf thymus DNA μM: A = 0, B = 38, C = 76, D = 152, E = 228.

With excess EN over Cu(II), the oxidation of EN accelerates with time. The lag period, the time required to achieve "maximal" rate of oxidation, increases with the relative amount of complex (Table I). This kinetic behaviour suggests that the rate determining step in the oxidation of $(EN)_2$-Cu(II) is the reduction of free Cu(II).

When [EN] exceeds [Cu(II)] the fraction of EN oxidized to adrenochrome decreases with [EN] (Table I). This is probably attributable to the binding of free Cu(II) by EN oxidation products, since the addition of Cu(II) reinitiates oxidation of EN after the reaction has stopped, while the addition of an EN equimolar concentration of adrenochrome to a reaction mixture which contains 50% complexed copper stops EN oxidation completely (data not shown).

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

Oxidation of the $(EN)_2$-Cu(II) complex by H_2O_2 is not affected by SOD or by OH radical scavengers such as PEG or mannitol. Sodium chloride enhances the copper catalyzed oxidation of EN. (Table I) This is probably not due to the increase in the ionic strength, since it is specific for chlorides (data not shown).

Ternary Complexes with $(EN)_2$-Cu(II)

EN-copper and EN-iron complexes form ternary complexes with various biological molecules and this complexation may change the redox potential of the CA. We noticed that the addition of BSA increases both the initial rate of oxidation and the amount of adrenochrome produced (Table I). Adsorption of the complex to *E. coli* cells slows down the initial rate of oxidation but has no effect on the amount of product (results not shown). While complexation with DNA slows down the initial rate of oxidation but increases the maximal rate of oxidation and the amount of adrenochrome produced.

Oxidation of the EN-Cu(II)-DNA Complex and Clevage of the DNA.

$(EN)_2$-Cu(II) binds to calf thymus or λ-DNA forming a mixed ternary complex with characteristic spectral properties. The difference spectra of the complexes formed with

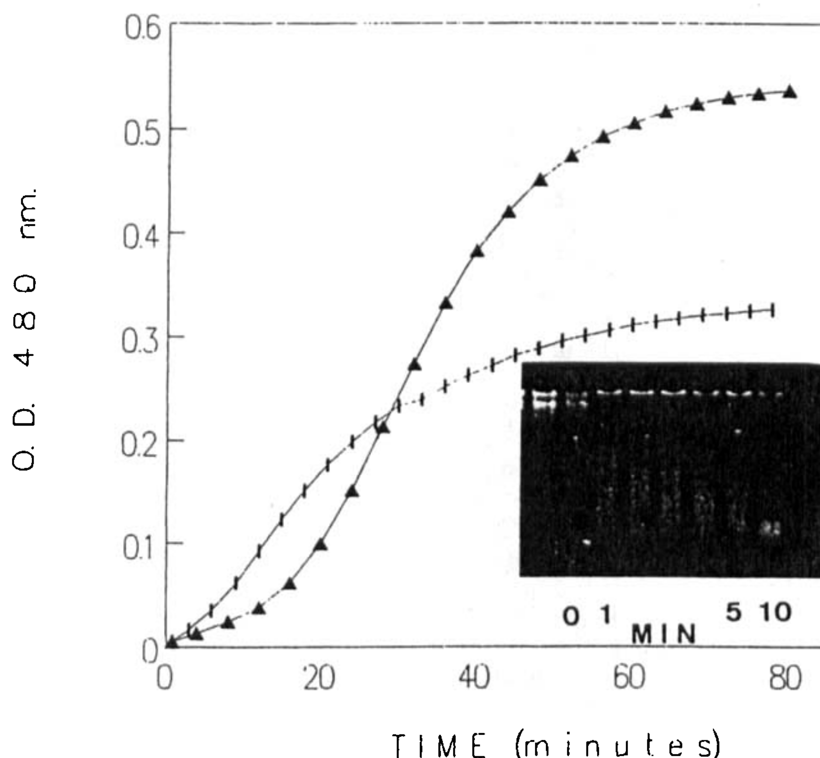

FIGURE 3. DNA cleavage and oxidation of the EN-Cu-DNA complex. EN 0.2 mM; Cu(II) 0.01 mM; H_2O_2 1 mM; calf thymus DNA 76 μM. Adrenochrome production |---| without DNA, ▲---▲ DNA added. Insert: DNA cleavage during the first 10 minutes of EN oxidation.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

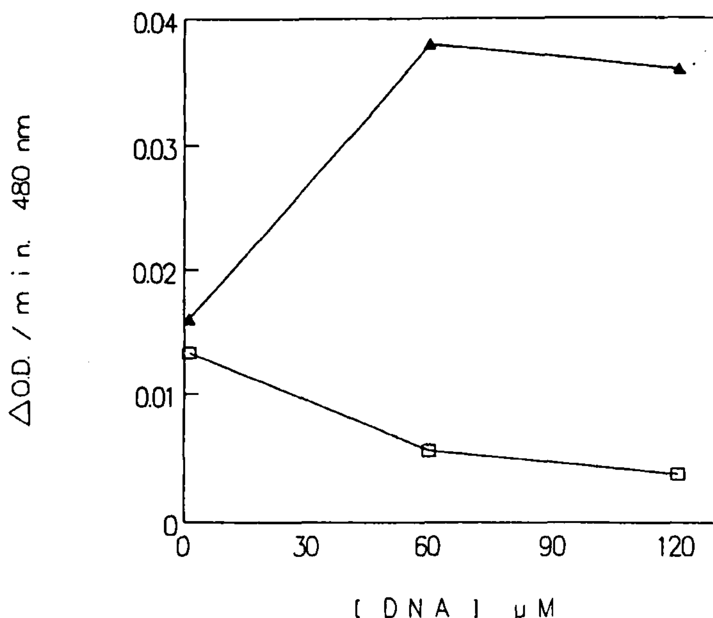

FIGURE 4. Effect of [DNA] on the oxidation rate of $(EN)_2$-Cu(II) complex. EN 0.6 mM; Cu(II) 0.02 M; H_2O_2 2 mM; calf thymus DNA 0, 60 and 120 μM. □---□ " initial " rate (ΔOD 0-1 minute) ▲---▲ "maximal" rate.

increasing [DNA] read against blanks of EN with the appropriate amount of calf thymus DNA show isobestic points at 235 and 265 nm (Figure 2). The ternary complex has a difference spectrum minimum at 251 nm with an apparent molar extinction coefficent of 12000. Adding increasing amounts of calf thymus DNA to a fixed amount of $(EN)_2$-Cu(II) and monitoring the decrease of the absorption at 251 nm, shows that the molar ratio of $(EN)_2$Cu(II) per DNA-phosphate at saturation is in the order of 1:20. The concentration of DNA was determined spectrophotometrically assuming that the extinction coefficient of DNA per nucleic acid phosphate at 258 nm is 6875 (M^{-1},cm^{-1}).

DNA slows down the initial rate of $(EN)_2$-Cu(II) oxidation by H_2O_2 and changes the shape of the oxidation curve, which becomes sigmoidal. (Figure 3) The oxidation rate increases with time and the maximal rate which is achieved exceeds the rate achieved in the absence of DNA. (Figure 4) The insert in Figure 3 relates EN oxidation with the time course of DNA degradation. It can be seen that the DNA has been cleaved to fragments smaller than 125 base pairs, during the slow phase of the EN oxidation curve, while only a small fraction of the EN present has been oxidized. EN oxidation is slow as long as highly polymerized DNA is present and accelerates during DNA degradation.

The correlation between EN oxidation rate and the clevage of the DNA in ternary complex with $(EN)_2$-Cu(II) was studied by varying the EN/Cu(II) ratio, and thus varying the rate of EN oxidation while [Cu(II)] and [H_2O_2] were kept constant. The concentrations of copper and H_2O_2 used did not produce measurable double strand breaks in the absence of EN. Figure 5 shows the rates of DNA cleavage when the concentration of complexed copper is varied from 16% (EN/Cu = 1/1) to 48% (EN/Cu = 20/1). It is apparent that the rate of DNA cleavage decreases with the

RIGHTSLINK

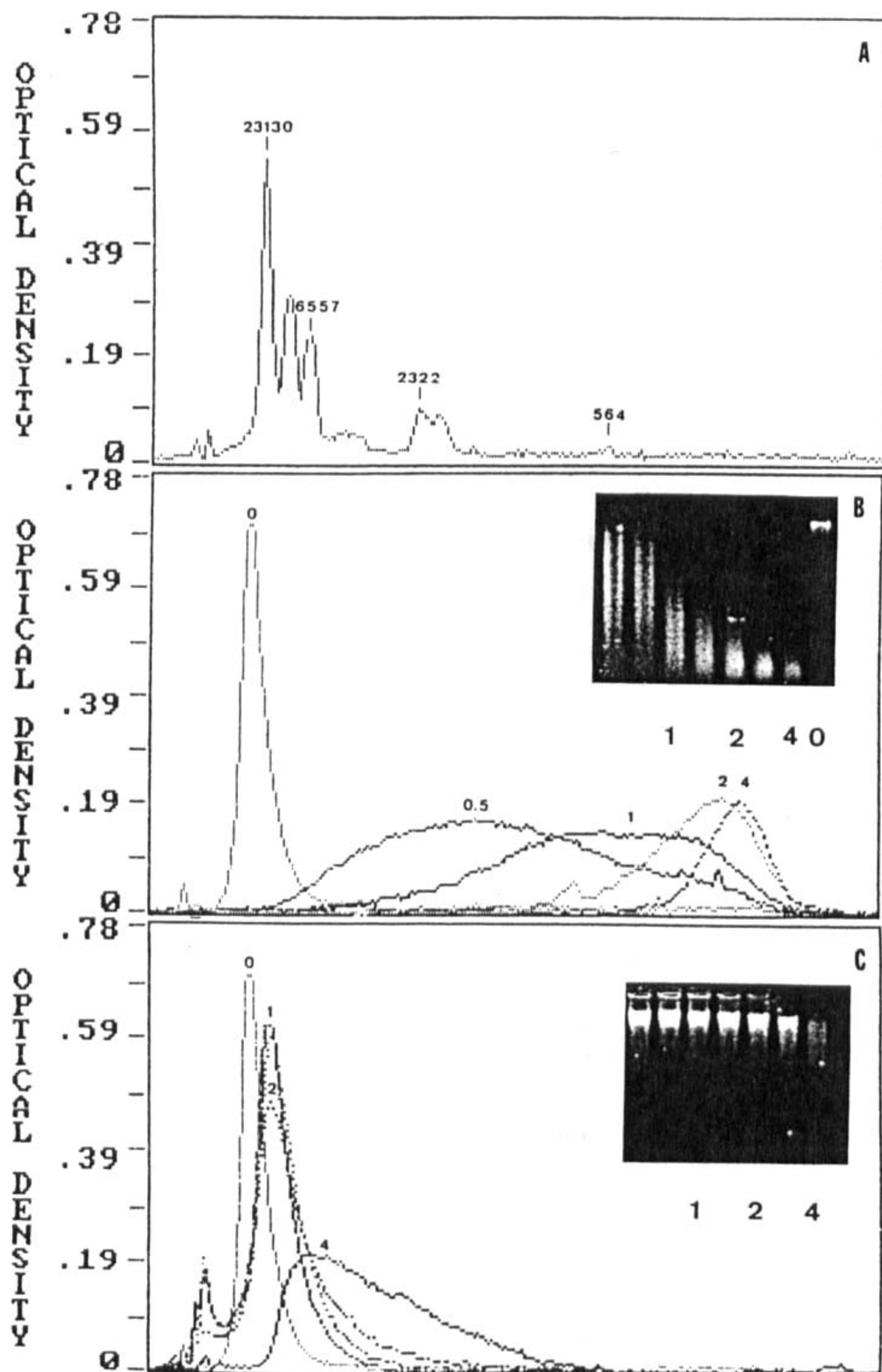

FIGURE 5 λ DNA cleavage: effect of EN/Cu(II) ratio. Electrophoretic profiles and optical scans. Cu(II) 0.1 mM; H_2O_2 1 mM; λ DNA 0.06 mM. Incubated at 37° for 0, 0.5, 1, 2 and 4 minutes. A: Hind III digest M.W. marker, B: EN/Cu(II) ratio 1:1, C: EN/Cu(II) ratio 20:1.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

increase in the relative amount of complexed copper. The rate of DNA cleavage is therefore proportional to the oxidation rate of EN. The same correlation was found with norepinephrine and dopamine-copper complexes. As a consequence of the H_2O_2-dependent oxidation of the ternary complexes the DNA is degraded in a similar manner to the thiol or H_2O_2-dependent degradation of DNA by doxorubicine-iron complexes.[18,19] We compared the rates of λ-DNA degradation and cleavage with $(EN)_2$-Cu(II), Cu(II)+ascorbate and o-phenanthroline-Cu(II) in the presence of 1 mM H_2O_2. $(EN)_2$ – Cu(II) was more active, per atom copper, than Cu(II)+ascorbate but somewhat less active than o-phenanthroline-copper (results not shown).

DISCUSSION

The initiating step in the oxidation of catecholamines in the presence of oxygen seems to be the metal catalyzed electron transfer from the mono anion of the CA to molecular oxygen forming a o-semiquinone and O_2^- which is scavanged by the catecholamine. The o-semiquinone radicals decay rapidly by disproportionation to give catechol and o-quinone.[20] When H_2O_2 is present and the reduced metal reacts with it in a Fenton-like reaction, hydroxyl radicals or higher oxidation states of the metal may be produced instead of O_2^-.

The results presented in this paper indicate that the oxidation of epinephrine or dopamine-Cu(II) complexes by H_2O_2 is mediated by free copper ions and the rate determining reaction is their reduction. When Cu(II) is in excess over EN the reaction proceeds to completion. However when [EN] is increased while Cu(II) is kept constant the oxidation rate and the relative amount of adrenochrome produced decrease progressively even though H_2O_2 is present in excess. We suggest that the reaction stops before completion because free Cu(II) ions bind to the reaction products. This assumption is supported by the observation that EN oxidation is inhibited by adrenochrome and can be reinitiated by the addition of copper.

NaCl and KCl accelerate the copper catalyzed oxidation of EN.[8] We observed that the concentration of chlorides required to accelerate the reaction markedly has to be above 10 mM and the extent of enhancement increases with the concentration of free Cu(II) ions. The effect does not require oxygen. Moreover, in the presence of NaCl, EN is oxidized under anoxia even in the absence of H_2O_2. These observations suggest several possibilities. When H_2O_2 is present hypochlorite may be formed from NaCl.[21] Alternatively, either the presence of Cl^- stabilizes the monovalent copper ions or it gives rise to copper species which are reduced faster than Cu(II) ions and may also serve as an "electron sink" for the oxidation of EN in the absence of O_2 and H_2O_2.

We have shown that the $(EN)_2$-Cu(II) complex may form redox active ternary complexes with small molecules such as ATP and with various macromolecules such as BSA, components on the surface of *E. coli* cells or DNA. The optical and catalytic properties of the ternary complex with DNA were studied in some detail. Addition of DNA to a reaction mixture which contained about 60% epinephrine-complexed copper and a large excess of free EN, slowed down the initial rate of EN oxidation. Oxidation of EN accelerated while the DNA was being degraded. The maximal oxidation rate and the amount of adrenochrome produced were higher than in the absence of DNA. The effect of DNA on the initial rate of oxidation may be due to chelation of free copper ions by the DNA and/or to a change in the stability constant or the one electron redox potential of copper in ternary complex with DNA. The maximal rate of oxidation achieved in the presence of DNA exceeds the rate achieved

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK

without DNA. This may indicate that copper bound to DNA fragments is reduced even faster than free copper, or alternatively, that the molecular and radical intermediates formed by the oxidation of EN bind Cu(II) ions and this binding inhibits their reduction, as already suggested. In the presence of DNA no such effect occurs, presumably, because the DNA cleavage products have a higher affinity for copper ions than EN oxidation products but nevertheless allow redox cycling of the copper. The rate of DNA cleavage is proportional to the rate of EN oxidation. This does not discriminate between oxygen derived radicals and quinone radicals as the damaging species. Although t-butanol (10 mM) was only slightly protective against DNA cleavage, it is possible that much higher concentrations are required to scavenge OH· formed in the vicinity of the target.[22] Therefore it is tempting to attribute the DNA breaks to the direct action of locally generated activated oxygen species which are expected to cleave DNA in solution more efficiently than quinone oxidation products.

Complexation of transition metal ions with the relatively non toxic catecholamines may lead to the formation of redox active ternary complexes with various biological macromolecules. All respiring aerobic organisms produced hydrogen peroxide which may initiate oxidation of the complexed cathecholamine with the accompanied degradation of the ligand macromolecules. Although, *in vitro*, complexation with DNA slows down the oxidation of EN, in a living system it may target the $(EN)_2$-Cu(II) complex to sites within the cell where oxidation of the complex may produce irreversible damage. The redox potential of catecholamine radicals may limit their reactivity in peroxidase catalyzed NAD(P)H or GSH mediated oxygen activation *in vitro*.[13] However since the EN-copper complex is bactericidal under anoxia and in the absence of added H_2O_2[14] it seems possible that the ternary complexes may act as oxidation-reduction catalyst in peroxidase systems *in vivo*.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

Acknowledgements

This study was conducted under the auspices of grants from the Council of Tobacco Research and the Israeli Academy of Science.

References

1. D.G. Graham, S.M. Tiffany, W.R. Bell and W.F. Gutknecht (1978) Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopaine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells *in vitro*. *Molecular Pharmacology*, **14**, 644–653.
2. M.M. Wick (1978) Dopamin: a novel antitumor agent active against B-16 melanoma in vivo. *Journal of Investigative Dermatology*, **71**, 163–164.
3. P.K. Singal, N. Kapur, K.S. Dhillon, R.E. Beamish and N.S. Dhalla (1982) Role of free radicals in catecholamine-induced cardiomyopathy. *Canadian Journal of Pharmacology*, **60**, 1390–1397.
4. M.M. Wick (1982) Therapeutic effect of dopamine infustion on human malignant melanoma. *Cancer Treatment Reports*, **66**, 1657–1659.
5. P.S. Dasgupta and T. Lahiri (1987) Antitumor effect of i.p. dopamine in mice bearing Ehrlich ascites sarcoma. *Cancer Research Clinical Oncology*, **113**, 363–368.
6. F.S. Archibald and C. Tyree (1987) Manganese poisoning and the attack of trivalent manganese upon catecholamines. *Archives of Biochemistry and Biophysics*, **256**, 638–650.
7. S.L. Jewett, L.J. Eddy and P. Hochstein (1989) Is the autoxidation of catecholamines involved in ischemia-reperfusion injury? *Free Radical Biology and Medicine*, **6**, 185–188.
8. P. Chaix, J. Chauvet and J. Jezequel (1950) Etude cinetique de l'oxydation de l'adrenaline en solution tampon-phosphate. *Biochimica et Biophysica Acta*, **4**, 471–483.
9. G. Litwack (1962) Characteristics of a ferrous iron complex of L-epinephrine. *Life Siences*, **10**, 509–513.

RIGHTSLINK

10. E. Walaas, O. Walaas and S. Haavaldsen (1963) Spectrophotometric and electron-spin resonance studies of complexes of catecholamines with Cu(II) ions and the interaction of ceruloplasmin with catecholamines. *Archives of Biochemistry and Biophysics*, **100**, 97–109.
11. R.K. Boggess and R.B. Martin (1975) Copper (II) chelation by Dopa, Epinephrine, and other catechols. *Journal of the American Chemical Society*, **97**, 3076–3081.
12. B. Kalyanaraman, C.C. Felix and R.C. Sealy (1984) Peroxidatic oxidation of catecholamines. A kinetic electron spin resonance investigation using the spin stabilization approach. *The Journal of Biological Chemistry*, **259**, 7584–7589.
13. J. O'Brien (1988) Radical formation during the peroxidase catalyzed metabolism of carcinogens and xenobiotics; the reactivity of these radicals with GSH, DNA and unsaturated lipid. *Free Radical Biology and Medicine*, **4**, 169–183.
14. J. Aronovitch, D. Godinger and G. Czapski (1990) Bactericidal activity of catecholamine-copper complexes. *Free Radical Research Communications*, this proceeding.
15. A. Rotman, J.W. Daly and C.R. Creveling (1976) Oxygen dependent reaction of 6-hydroxydopamine, 5,6-dihydroxytryptamine and related compounds with proteins *in vitro*: a model for cytotoxicity. *Molecular Pharmacology*, **12**, 887–889.
16. T. Maniatis, E.F. Fritch and J. Sambrook (1982) Isolation of bacteriophage. In *Molecular Cloning*, Cold Spring Harbor Laboratory Publications, pp. 75–96.
17. J. Aronovitch, D. Godinger, A. Samuni and G. Czapski (1987) Ascorbic acid oxidation and DNA scission catalyzed by iron and copper chelates. *Free Radical Research Communications*, **2**, 241–258.
18. H. Eliot, L. Gianni and C. Myers (1984) Oxidative destruction of DNA by the adriamycin-iron complex. *Biochemistry*, **23**, 928–936.
19. J. Muindi, B.K. Sinha, L. Gianni and C. Myers (1985) Thiol-dependent DNA damage produced by anthracyclin-iron complexes. The structure-activity relationships and molecular mechanisms. *Molecular Pharmacology*, **27**, 356–365.
20. P. Gee and J. Davison (1985) 6-Hydroxydopamine does not directly reduce molecular oxygen but requires a coreductant. *Archives of Biochemistry and Biophysics*, **231**, 164–168.
21. K. Frenkel, F. Blum and W. Troll (1986) Copper ions and hydrogen peroxide form hypochlorite from NaCl thereby mimicking myeloperoxidase. *Journal of Cellular Biochemistry*, **30**, 181–193.
22. G. Czapski (1984) On the use of OH· scavengers in biological systems. *Israel Journal of Chemistry*, **24**, 29–32.

Accepted by Prof. G. Czapski

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/09/11
For personal use only.

RIGHTSLINK