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Effect of Purine Nucleoside Phosphates on OH-Radical Generation By Reaction of Fe^{2+} With Oxygen

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The influence of various purine nucleotides, nucleosides and nucleoside phosphates on the generation of OH-radicals by the reaction of Fe^{2+} with oxygen was investigated. Coumarin-3-carboxylic acid was used as a fluorescent detector of OH·. Nucleoside triphosphates caused the enhancement of OH· production due to chelation of ferrous ion by the phosphate moiety. About 30% of produced OH· are intramolecularly scavenged by the nucleoside moiety of the chelator molecule. Nucleoside diphosphates cause a slight enhancement of OH· yield. Nucleotides, nucleosides and nucleoside monophosphates decrease the OH· production.

Rate constants of reaction between OH· and nucleoside derivatives were determined from the competitive scavenging of OH radicals, produced by oxidation of Fe^{2+}-EDTA complex. Derivatives of guanosine and xanthine are more efficient scavengers in comparison to adenine and inosine. Phosphate groups do not affect the constant of reaction of nucleoside with OH·. Our results suggest that the yield of OH· in the presence of the nucleotide derivatives is determined by chelation of ferrous with polyphosphates and preferential OH· scavenging by the organic portion of molecule.

We propose that the generation of active oxygen intermediates in the reaction between nucleoside triphosphate complexes of iron and molecular oxygen is involved in iron-related cellular injury.

Keywords: Hydroxyl radicals, iron, nucleoside phosphates, fluorescence, coumarin

INTRODUCTION

The reaction Fe^{2+} with oxygen (autoxidation) may be a significant mechanism of OH· production and the iron participation in radical toxic processes.[1] Unlike Fenton or Haber-Weiss reactions, it does not require any additional conditions for the prior generation of active oxygen intermediates (superoxide and hydrogen peroxide). However, the investigation of this process has attracted much less attention. Because of this, there is a lot of controversial data about OH· generation during ferrous autoxidation in the literature. For example, OH· production during ferrous autoxidation was not detected by DMPO spin trapping, but was found under the same conditions in the Fenton reaction.[2]. This difference, however, can be explained as a result of DMPO-OH oxidation by ferrous-mediated processes.[3]

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An inhibition of deoxyribose degradation by ADP and ATP was described for ferrous autoxidation,[4] but not for the Fenton reaction.[5]

We detected OH· production by ferrous autoxidation and effect of polyphosphates at physiological conditions using coumarin fluorescent probe.[6, 7] The enhancement of OH· production by tripolyphosphate $P_3O_{10}^{5-}$ was explained as a result of Fe^{2+} chelation.[7] It caused an alteration of the reaction mechanism from two-electron to one-electron oxygen reduction and subsequent increasing of $O_2^{\cdot-}$ and OH· production. A similar effect of purine nucleoside triphosphates was also mentioned,[6] but the scavenging ability of the organic part of the molecules was not quantitated. Nucleoside diphosphates are also known as complexing agents for Fe^{2+}.[8, 9] However, any significant enhancement of OH· production by guanosine diphosphate GDP was not detected by the fluorescent method.[7].

In this paper we investigate the OH· production by the Fe^{2+} autoxidation in the presence of four different purine phosphates: adenine, guanine, inosine and xanthine. Coumarin-3- carboxylic acid (CCA) was used as a fluorescent probe for detection of OH·.[10] This method is based on the measurement of the fluorescence of 7-hydroxycoumarin-3-carboxylic acid (7 OHCCA), produced by the reaction between CCA and OH·.

Our previous focusing on the GTP effect[6] was based on the possible connection between chelation of ferrous by this nucleoside and signal transduction.[8] However, ATP is present *in vivo* in much higher concentrations and is considered as the main triphosphatic chelator of free iron in cells.[11] Inosine triphosphate (ITP) has been identified as a possible clastogenic factor in the blood of accidentally irradiated patients,[12] and can also be considered as a potential ferrous chelator.

Comparison of the effect of various purine nucleotide compounds allows us to determine the correlation between the molecular structure of chelator and its involvement in the Fenton-linked OH-radical production.

MATERIALS AND METHODS

All the reagents were purchased from Aldrich-Sigma and used without any additional treatment; $FeSO_4 \cdot 7H_2O$ was recrystallized twice prior to usage. Reactions were performed at 37°C in 20 mM potassium phosphate buffer (pH = 7.40), which was prepared using Milli-Q water (18 M Ω·cm) with further treatment through Chelex-100.

Production of OH· was determined from the fluorescence (emission at 450 nm, excitation at 400 nm) of 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA), which was generated from the coumarin-3-carboxylic acid (CCA) probe and OH·. Fluorescent measurements were performed using SPF-500 spectrofluorometer (SLM Instrument Co., Urbana, IL, USA). In order to avoid an inner filter effect the absorbence spectra were recorded by DW-2000 spectrophotometer (SLM Instrument Co., Urbana, IL, USA) for all the samples prior and after experiment. The absorption at 400 as well as 450 nm did not exceed 0.05 for all the used samples.

Real-time kinetic measurements were performed in kinetic mode. The production of 7-OHCCA was calculated from a comparison of sample fluorescence and the standard 7-OHCCA solution. The rate constants of the reaction of nucleoside phosphates with OH· were determined from the competitive scavenging of OH radicals, produced by 0.1 mM Fe^{2+}-EDTA complex and detected by 0.1 mM CCA. The constants were calculated from the linear interpolation of reciprocal values of 7-OHCCA production in the concentration range 0–0.3 mM. The slopes of the line were compared with data on ribose and deoxyribose, which constants of the reaction with OH· ($1.5 \cdot 10^9$ and $1.9 \cdot 10^9$ $M^{-1} \cdot s^{-1}$[13]) were used as reference points.

RESULTS

The addition of purine nucleoside triphosphates increases the yield of 7-OHCCA, produced in the

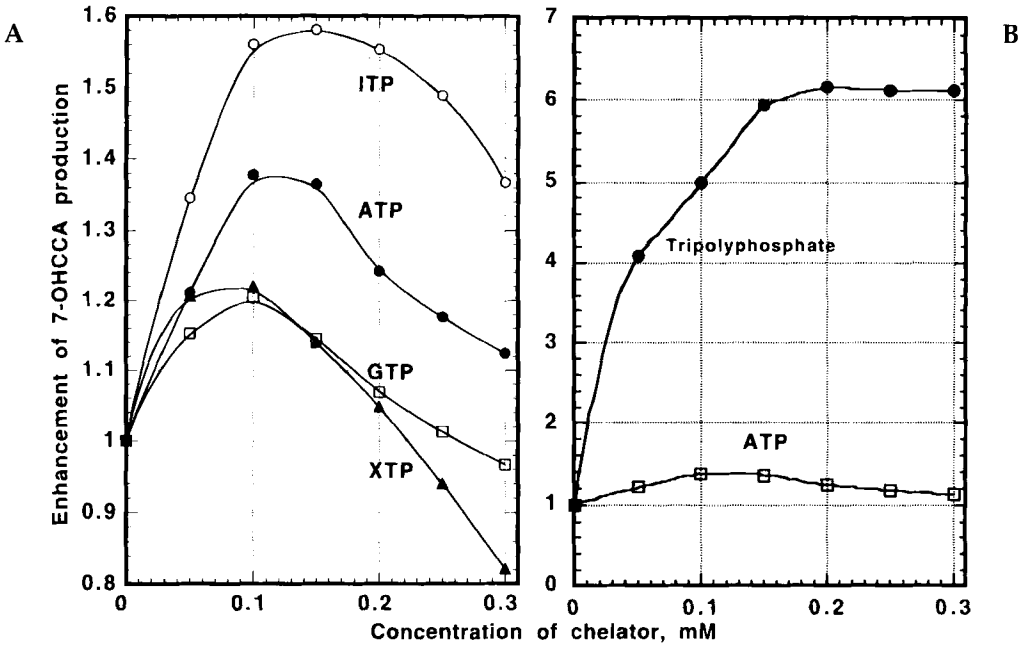

FIGURE 1 Enhancenent of 7-OHCCA production, generated by oxidation of 0.1 mM Fe^{2+} in presence of 0.1 mM CCA, by nucleoside triphosphates (1A) and tripolyphosphate (1B).

reaction of Fe^{2+} with O_2 (Fig. 1). The enhancement is maximal for the equimolar mixtures of Fe^{2+} and triphosphate in the presence of 0.1 mM Fe^{2+} and CCA. The level of enhancement is highest for ITP (about 60%) and lowest for xanthine triphosphate (XTP) and GTP (20%). These values are significantly lower in comparison with effect of other ferrous chelators. For example, the enhancement of 7-OHCCA production by tripolyphosphate is one order of magnitude higher under these conditions (Fig. 1B).

The influence of nucleotides, nucleosides, and their mono- and diphosphates on the 7-OHCCA production was also tested in the presence of 0.1 mM concentrations of Fe^{2+} and CCA. Three compounds (guanine, xanthine and xanthosine) could not be used because of their low solubility. All analyzed nucleotides, nucleosides, and nucleoside monophosphates decrease the 7-OHCCA yield, as shown in Figure 2A for adenine derivatives. The dependence of reciprocal values of the 7-OHCCA production on the nucleoside derivative concentration is linear (Fig. 2B). It confirms the action of these compounds as OH· scavengers.[13]

Nucleoside diphosphates also decrease the 7-OHCCA production. However, the dependence of reciprocal 7-OHCCA production on the concentration of nucleoside diphosphates is not linear (Fig. 2B). Triphosphates show the same type of dependence, which is caused by the chelation of Fe^{2+}. It suggests a possible action of nucleoside diphosphates as ferrous chelators.

To confirm this effect, the reaction was performed with 1 mM CCA and 0.01 mM Fe^{2+} to reduce the competitive OH· scavenging by the other molecules. Under these conditions the nucleoside diphosphates show an enhancement of 7-OHCCA production, as shown in Figure 3 for guanosine derivatives.

The level of 7-OHCCA production enhancement by nucleoside triphosphates in presence of high CCA concentration is also significantly increased. Under these conditions it is similar to yields obtained with tripolyphosphate (Fig. 4).

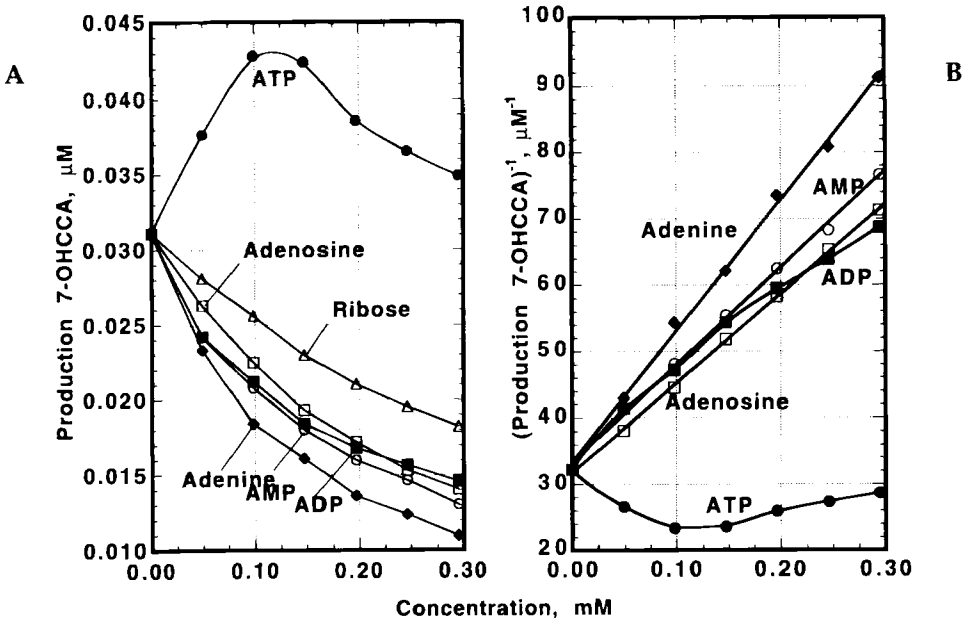

FIGURE 2 Effect of different derivatives of adenine on production of 7-OHCCA (A) and its reciprocal value (B) by 0.1 mM Fe^{2+} in presence of 0.1 mM CCA.

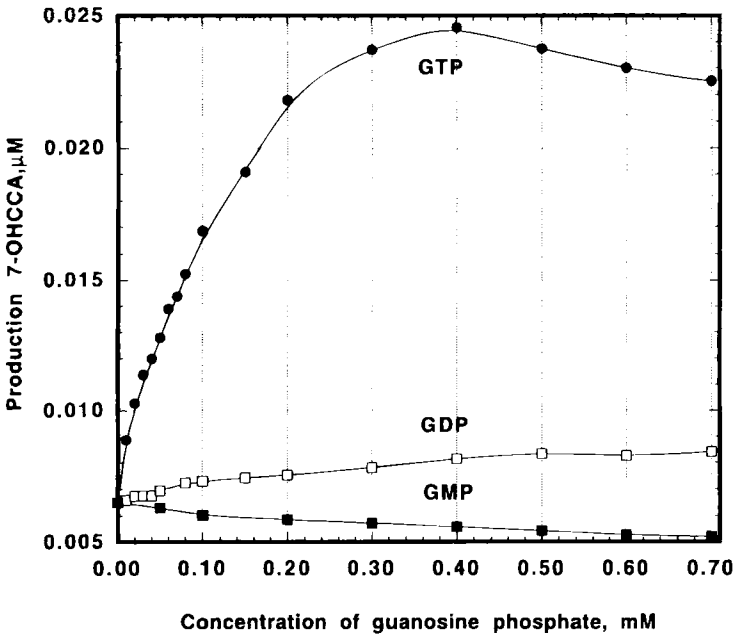

FIGURE 3 Production of 7-OHCCA by 0.01 mM Fe^{2+} in presence of guanosine phosphates; detection by 1 mM CCA.

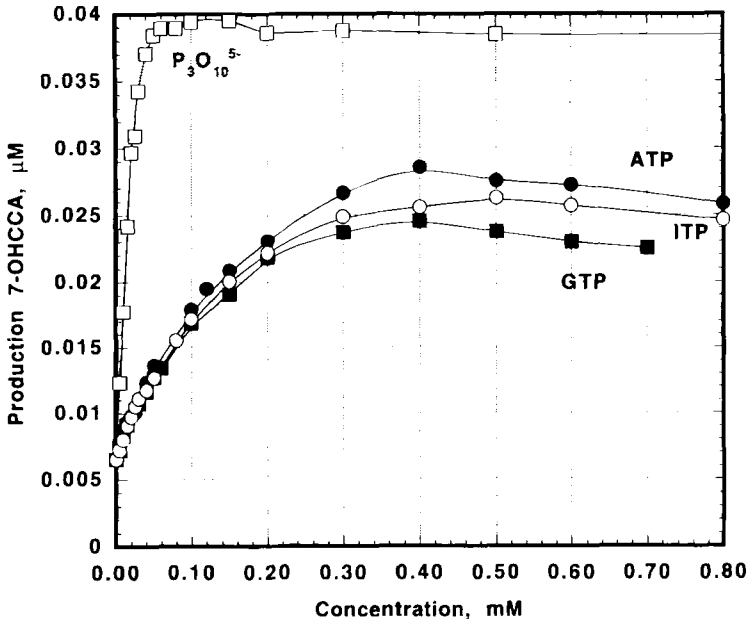

FIGURE 4 Comparison of enhancement of 7-OHCCA production by nucleoside triphosphates and tripolyphosphate for 0.01 mM Fe^{2+} and 1 mM CCA.

Nucleoside monophosphates still act as scavengers, decreasing the OH· production (Fig. 3).

We used the competitive OH· scavenging between CCA and nucleoside derivatives to determine the rate constants of reactions between nucleoside phosphates and OH·. In this study OH· were generated by oxidation of the premixed 0.1 mM Fe^{2+} and EDTA in the presence of 0.1 mM CCA. Complex of Fe^{2+} with EDTA was chosen because of its stability to dissociation (pK = 14.33[14]). The results are shown in figure 5. Data on ribose and deoxyribose[13] were used as the reference points.

DISCUSSION

The OH· production in the reaction of ferrous with oxygen is enhanced by nucleoside triphosphates due to the ferrous chelation by the tripolyphosphate (inorganic) moiety of molecule.[6] Difference in the enhancement levels between nucleoside triphosphates (Fig. 1) can be explained as the result of OH· scavenging by nucleoside (organic) moiety of molecule. Because the chelator molecule is able to react with OH·, we consider two simultaneous processes of OH· scavenging. Part of the OH· reacts in the site of generation with the same nucleoside molecule, which chelates ferrous ion, as is shown in Figure 6. This process can be considered as an intramolecular reaction. The rest of OH· reacts in solution by intermolecular reactions with other scavenger molecules. Competition by CCA and nucleoside phosphates in the intermolecular process determines the yield of 7-OHCCA.

In order to separate the intramolecular and intermolecular reactions, the reaction conditions were modified. We used the excess of fluorescent probe (1 mM CCA and 0.01 mM Fe^{2+}) to minimize the influence of intermolecular scavenging by nucleoside phosphates. The chelation of iron with EDTA allows us to create the opposite situation, where the intramolecular reaction between OH· and nucleoside triphosphates is not possible, and the yield of 7-OHCCA is determined only by intermolecular scavenging.

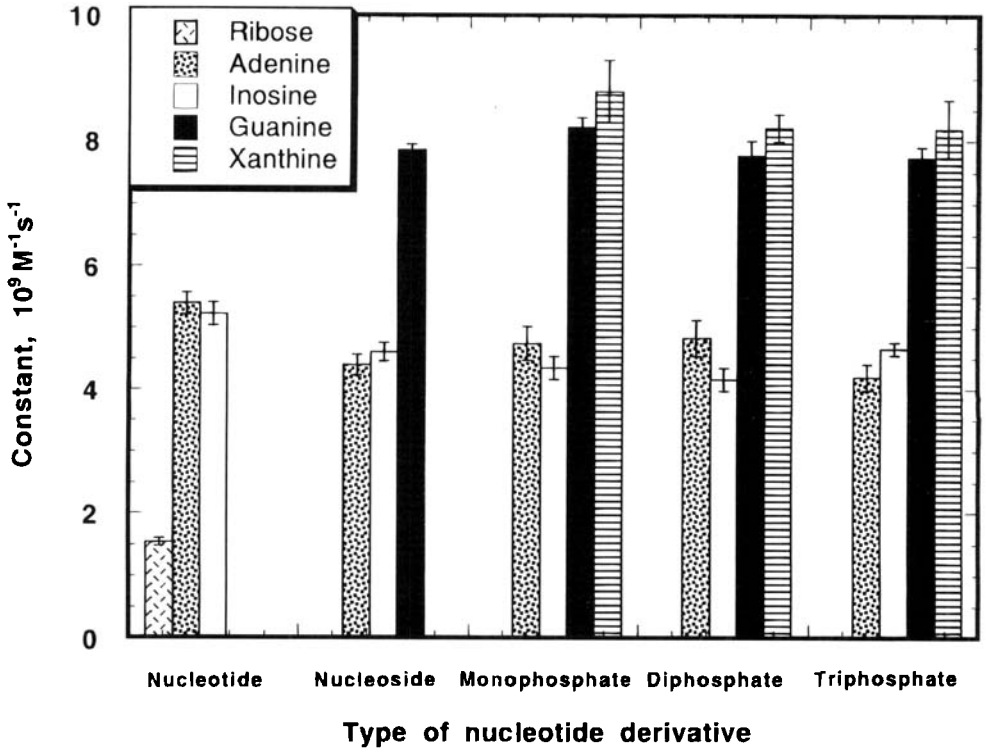

FIGURE 5 Rate constants of reaction of various nucleotide derivatives with OH radical.

Under conditions of minimized intermolecular scavenging, nucleoside triphosphates cause a similar yield of OH·, as it is seen in Figure 4. The minor differences in the values of 7-OHCCA emerge at higher concentrations of nucleoside triphosphates (~0.3 mM), when competition of nucleoside phosphates with CCA for the intermolecular scavenging becomes significant.

Enhancement of 7-OHCCA production by nucleoside triphosphates with 0.1 mM concentrations of reagents was substantially lower in comparison with the effect of inorganic tripolyphosphate (Fig. 1). Depletion of the intermolecular OH· scavenging significantly increases the 7-OHCCA production by nucleoside triphosphates, making it comparable with inorganic tripolyphosphate (Fig. 4). Maximal enhancement of 7-OHCCA production at 0.4 mM of nucleoside triphosphates is 60–70% of the tripolyphosphate effect. This difference suggests, that about one

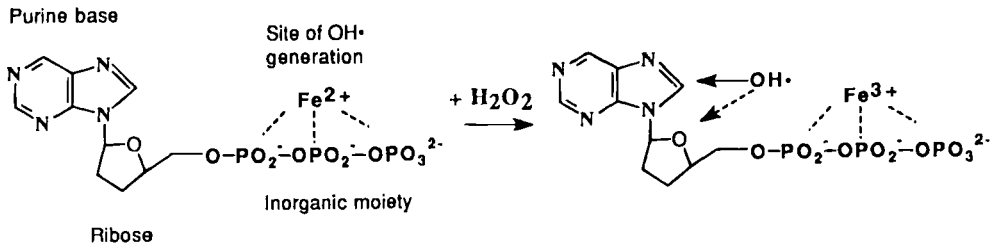

FIGURE 6 Intramolecular scavenging of OH· by nucleoside moiety of chelator.

third part of OH· is consumed by nucleoside triphosphates in the intramolecular reaction.

Chelation of ferrous ion by strong chelator EDTA excludes its binding to nucleoside phosphates. The participation of nucleoside phosphates in the intramolecular OH· scavenging is not possible under these conditions. Their influence on the total yield of 7-OHCCA production is determined by intermolecular competition for OH· between nucleoside triphosphates, CCA, and other components of the solution. In the presence of EDTA, nucleoside triphosphates. do not show any enhancement of OH· production, acting only as scavengers. Under these conditions the influence of mono-, di- and triphosphate of nucleoside is the same, which is reflected in the equal values of constants in Fig. 5. These data support the requirement of ferrous chelation for the enhancement of OH· production.

Competitive scavenging in the presence of EDTA allowed us to determine the rate constant of the reaction between nucleoside phosphates and OH·. This method of constant determination was proposed for the deoxyribose and benzoate assays.[15] Although the reaction mixture is complicated (Fe^{2+}-EDTA, Fe^{3+}-EDTA, CCA, nucleoside derivatives, and products of their degradation), the addition of nucleoside phosphates does not affect total kinetics of OH· production in the presence of EDTA. It allows us to compare the OH· scavenging by the nucleoside phosphates with data on ribose and deoxyribose using the fixed solution composition.

Figure 5 shows the rate constants for the reaction of purine nucleosides and their phosphates with OH·. Derivatives of xanthine and guanosine are more efficient OH· scavengers, than adenine and inosine. The values of constants for the nucleoside, its mono-, di- and triphosphates are equal. It suggests, that phosphate groups of nucleoside phosphates do not interact with OH·. Our previous observations also demonstrated the significance of the organic part of the molecule for OH· scavenging.[6] These results are in agreement with the prevailing location of the radical DNA damage on the base part rather than phosphate.[13, 16]

Conversion of nucleotides into nucleosides reduces their constants of reaction with OH· (cf. data on adenine with adenosine derivatives and hypoxanthine with inosine). On the contrary, OH· scavenging by equimolar mixture of ribose and nucleotide is additive (data not shown). This effect of ribose binding is probably related to lower constant of OH· scavenging by ribose (cf. data for ribose with other nucleosides shown on Fig. 5). The decreasing of the OH· scavenging after the ribose binding suggests the preferential OH· attack of base portion of the molecule in nucleosides and nucleoside phosphates. Equal values of constants for nucleosides and their phosphates (Fig. 5) also support the irrelevance of ribose for OH· scavenging in nucleosides. Otherwise, phosphorylation of ribose should affect the ability of the molecule to react with OH·.

The preferential OH· scavenging by nucleotide part of the molecule presumes the dependence of the OH· scavenging constant on the nucleotide chemical structure. Derivatives of disubstituted purines guanine (2-amino-6-hydroxypurine) and xanthine (2,6-dihydroxypurine) are more efficient OH· scavengers in comparison with subsequent monosubstituted purines adenine (6-aminopurine) and hypoxanthine (6-hydroxypurine, base of inosine phosphates). This variance is mainly responsible for different levels of the OH· production enhancement by nucleoside triphosphates at conditions of competition between probe and chelator (Fig. 1).

Intramolecular reaction between OH· and nucleoside triphosphate in the ferrous chelates is not a unique example of site-specific OH· scavenging. Our data show, that the efficiency of scavenging can increase due to the interaction of the scavenger molecule with site of OH· generation. For example, when OH· are produced by the oxidation of Fe^{2+}-EDTA complex, all four adenosine derivatives show the same scavenging (Fig. 5).

However, when OH· are produced by oxidation of unchelated Fe^{2+}, AMP and ADP showed a lower 7-OHCCA production in comparison with adenosine (Fig. 2A). This effect is the result of electrostatic interaction between negatively charged AMP or ADP molecules and Fe^{2+}, which is the site of OH· generation. It makes them more efficient scavengers in comparison with the neutral adenosine molecule.

The effect of ribose on the production of OH· by Fe^{2+} oxidation is another example of site specific scavenging. Ribose is less efficient OH· scavenger, than adenine (Fig. 2). However, the difference between these two compounds is lower, than it should be expected from a 3.6-fold difference of the rate constants (Fig. 5). The explanation of this effect is the possible ferrous chelation by ribose, which increases its scavenging ability. The formation of similar ferrous complexes was mentioned for deoxyribose.[17] The destruction of this complex by adenosine phosphates (together with OH· scavenging) can be a possible reason of inhibition of deoxyribose degradation by ADP and ATP by ferrous autoxidation, described in.[4] Chelation of iron with EDTA excludes its binding to ribose, as well as it was shown for deoxyribose.[15] Absence of an interaction between Fe-EDTA complex and ribose or deoxyribose allowed us to use these compounds as the reference points for the determination of the rate constants of reaction between nucleosides and OH·.

Nucleoside diphosphates were also used as ferrous chelators[4, 5, 8, 9] in chemical systems together with triphosphates. However, the effect of GDP on OH· production with 0.1 mM Fe^{2+} and CCA was similar to the action of scavenger GMP, but not to the ferrous chelator GTP.[6] More precise measurements show, that diphosphates cause a higher level of 7-OHCCA production in comparison with monophosphates, as seen in Figure 2 A for adenine derivatives. Reciprocal values of 7-OHCCA production in the presence of nucleosides diphosphates do not depend linearly on the concentration. The same effect occurs for nucleosides triphosphates (Fig. 2B), which chelate ferrous at these conditions. All these data suggest a possible involvement of nucleosides diphosphates not only in OH· scavenging, but also in OH· generation as ferrous chelators.

The conditions of minimized intermolecular OH· scavenging allowed us to prove this hypothesis. An addition of nucleoside diphosphates to 1 mM CCA and 0.01 mM Fe^{2+} causes a 30% enhancement of OH· production (Fig. 3). It is an order of magnitude less, than the effect of triphosphates under these conditions, and comparable to the difference between pyrophosphate and tripolyphosphate.[7] Subsequently, nucleoside diphosphates cause only a slight enhancement of OH· production, which is hidden by OH· scavenging at a low probe concentration (Fig. 2).

Investigation of the influence of polyphosphatic chelators on the ferrous autoxidation[6, 7] allows us to propose the mechanism of free iron action in cell damage processes. The release of free iron from intracellular storage sites is caused by various processes, e.g., oxidative stress,[18] action of xenobiotics[19] or superoxide radicals.[20] Apart from its normal biological pathways, free iron is involved in various pathological processes caused by oxidative stress and numerous diseases,[21] and most of free-radical injury is iron-related.[22] The mechanism of such injury generally considers an involvement of iron in the OH· production as a catalyst in Haber-Weiss reaction or in Fenton reaction.[23] However, both reactions require a simultaneous generation of other active species: superoxide anion $O_2^{\cdot-}$ and/or hydrogen peroxide H_2O_2 in cells. The source of these compounds generally remains unclear, especially when mechanisms of cell protection against their action (catalase, superoxide dismutase and glutathione peroxidase) are also considered.

The results of our investigations suggest another mechanism of iron-mediated OH· generation. It considers the generation of active oxygen compounds ($O_2^{\cdot-}$, H_2O_2 and OH·) in the reaction

between molecular oxygen and nucleoside triphosphate complexes of ferrous. After the release of iron from storage sites and its reduction, ferrous ions are chelated by ATP, GTP or ITP. Chelation of ferrous with nucleoside triphosphates decreases its ability to catalyze the Haber-Weiss reaction,[9] which might decrease the iron toxicity. However, these complexes can generate active oxygen intermediates ($O_2^{\cdot-}$, H_2O_2 and OH·) by the direct reaction with oxygen.[6] In the case of the triphosphate chelated ferrous, this reaction will be realized as a bimolecular one-electron reduction of oxygen:[7]

$$\left[Fe^{2+}(ATP)\right] + O_2 = \left[Fe^{3+}(ATP)\right] + O_{2\cdot} \quad (1)$$

$$\left[Fe^{2+}(ATP)\right] + O_{2\cdot^-} + 2H^+ = \left[Fe^{3+}(ATP)\right] + H_2O_2. \quad (2)$$

$$\left[Fe^{2+}(ATP)\right] + H_2O_2 = \left[Fe^{3+}(ATP)\right] + OH^- + OH\cdot. \quad (3)$$

The chelation of ferrous with nucleoside triphosphates is a necessary condition for the reactions (1–3). Without it, unchelated ferrous can react with oxygen only by the trimolecular reaction with simultaneous participation of two Fe^{2+}:[7]

$$2Fe^{2+} + O_2 + 2H^+ = 2Fe^{3+} + H_2O_2. \quad (4)$$

At a very low iron concentration the trimolecular process (4) will not be efficient and can be neglected in biosystems. Subsequently, unchelated ferrous can not be considered as a significant source of free radicals. On the contrary, bimolecular processes (1–3) occur regardless to free iron concentration. As a result, the chelation of free iron by nucleoside triphosphates converts it to a source of $O_2^{\cdot-}$, H_2O_2 and OH·, which are generated directly from O_2. Cellular damage caused by these radicals is the mechanism of free iron toxicity.

Nucleoside triphosphates can also play another role in the modulation of free iron toxicity. As shown above, the generation of OH· by the nucleoside triphosphate complex of ferrous is followed by intramolecular scavenging of 30% OH·. Subsequently, nucleoside triphosphates also react with OH· *in situ*, providing a partial protection of other biomolecules from radical damage. They also have high constants of reaction with OH·, and can be considered (together with glutathione and ascorbic acid) as significant cell protectors against OH·-mediated damage.

Conclusions

The effect of different purine phosphates on OH· production in the reaction of Fe^{2+} with oxygen was investigated. Nucleoside triphosphates cause the enhancement of OH· production due to the chelation of ferrous ion. About 30% of produced OH· are intramolecularly scavenged by nucleoside moiety of the chelator molecule. Nucleoside diphosphates are weaker chelators and cause only a slight enhancement of OH· yield, which is prevailed by OH· scavenging. Nucleotides, nucleosides and their monophosphates are OH· scavengers. Constants of interaction between OH· and nucleoside phosphates suggest an involvement of the nucleotide part of the molecule into a reaction with OH·. Phosphate groups are insignificant for OH· scavenging. The results allow us to propose a mechanism of iron-mediated radical damage, which can be involved in iron cellular toxicity.

Acknowledgments

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