Hypoxia-dependent Reduction of 1-(2-Nitro-1-imidazolyl)-3-methoxy-2-propanol by Chinese Hamster Ovary Cells and KHT Tumor Cells in Vitro and in Vivo

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SUMMARY

Incubation of Chinese hamster ovary cells and KHT murine fibrosarcoma tumor cells in the absence of oxygen with 1-[2-14C]nitro-1-imidazolyl)-3-methoxy-2-propanol, one of the most effective radiation sensitizers of hypoxic cells, results in the preferential reduction of 1-[2-14C]nitro-1-imidazolyl)-3-methoxy-2-propanol. The radioactivity associated with the acid-insoluble precipitate from cells incubated in nitrogen is about four times higher than that of cells incubated in air. When aqueous extracts of tissues of a C3H mouse bearing the KHT tumor, after i.p. injection with 1-[2-14C]nitro-1-imidazolyl)-3-methoxy-2-propanol, are analyzed, a reduction product is found in relatively higher yields in the tumor than in normal tissues. The relative radioactivity in the pellet from the tumor homogenate is also high in comparison with those of most normal tissues. These results provide suggestive evidence for a higher degree of hypoxia in the tumor than in most normal tissues. The formation of reduction products and their subsequent binding to macromolecules may explain the preferential toxicity of nitro compounds to mammalian cells under hypoxic conditions. These results suggest that some nitro compounds may be useful for the treatment of tumors having a high fraction of hypoxic cells even in the absence of radiation.

INTRODUCTION

The possible use of hypoxic cell radiation-sensitizing drugs in the radiation therapy of certain types of tumors is of considerable current interest (1). A basic assumption here is that the presence of a hypoxic cell fraction in the tumor makes it more resistant to radiation than if all cells were oxygenated. Studies in recent years have shown that a number of electron-affinic nitro compounds sensitize mammalian cells in vitro under hypoxic conditions to ionizing radiation (2, 3, 7). Very promising in vivo results have also been obtained with some of these compounds (6, 10, 18). Among these, a 2-nitroimidazole derivative, NIMP, appears to have the maximum potential for clinical use.

In the course of studies on the mechanism of the radiation sensitization of hypoxic cells by electron-affinic nitro compounds, it was observed that: (a) NIMP is more toxic to hypoxic cells than oxygenated cells (16, 17) and (b) preincubation of cells with NIMP under hypoxic conditions at 37° enhances the killing of the cells by radiation (G. F. Whitmore and S. Gulyas, unpublished data). To understand these effects at a molecular level, the fate of NIMP was investigated in CHO cells and in single-cell suspensions of KHT fibrosarcoma tumor cells in vitro. The results of these studies are described in this paper and show that under hypoxic conditions a specific reduction product of NIMP is preferentially formed. When KHT tumor-bearing C3H mice were given injections of NIMP and were analyzed for products, the concentration of this reduction product was found to be higher in the tumor than in most other tissues.

MATERIALS AND METHODS

Synthesis of 14C-Labeled NIMP (I). Following the procedure of Shildneck and Windus (19) S-methylisothio[14C]urea sulfate (II) was prepared from thiourea containing 0.1 mCi thio[14C]urea per mmole. Reacting II with aminoacetaldehyde diethylacetal, as described by Storey et al. (21), 2-amino[2-14C]imidazole sulfate (III) was obtained. Diazotization of III and subsequent reaction with sodium nitrite in the presence of CuSO4 and H2SO4 (5) yielded 2-nitro[2-14C]imidazole (IV). To prepare I, the procedure of Beaman et al. (4) with slight modification was adopted. This involved the heating of IV with 1,2-epoxy-3-methoxypropene in the presence of K2CO3. After boiling commenced, the heating was stopped. After 10 min the hot mixture was filtered. Upon refrigeration of the filtrate, yellowish-brown crystals formed. Generally, about 3.5 g of NIMP were obtained from 15.2 g of thiourea. Recrystallization of the crude product from hot, absolute ethanol afforded a yellowish-white crystalline product (m.p. 110-111°; λmax in isopropyl alcohol 315 nm; εmax 7000).

Infrared absorption spectrum of the synthetic sample was  

C7H11N4O4  
Calculated: C 41.79, H 5.47, N 20.89  
Found: C 41.52, H 5.30, N 20.62

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The tumor used in the study, the KHT fibrosarcoma (15) originally arose spontaneously in a male C3H mouse. The details of the procedure for the transplantation of the tumor have been described previously (22). It was transplanted routinely by injecting $2 \times 10^7$ cells s.c. into both flanks of 8- to 10-week-old male C3H/He Dub mice (about 20 g by weight). In 10- to 14 days, the tumor became 10 to 14 mm in diameter. For in vivo studies the drug [25 mg in 3 ml of phosphate-buffered saline (NaCl, 0.8%; KCl, 0.02%; KH$_2$PO$_4$, 0.02%; Na$_2$HPO$_4$, 0.115%)] was injected i.p. After 4 hr, the animal was killed, the organs of interest were removed, and the tissues were cut into small pieces, homogenized in 3 volumes of water at 0°, and centrifuged at 40,000 x g for 40 min. The supernatant was chromatographed as described below.

**Preparation of Single-Cell Suspensions of KHT Tumor.** The detailed procedures for the preparation of single-cell suspensions of the KHT tumor have been described previously (22). The method yielded about $3 \times 10^7$ cells/g of the tumor.

**Cell Culture.** The CHO cells used in this study have been routinely grown in suspension culture in α media containing fetal calf serum as described previously (16).

**Cell Extracts.** Either CHO or KHT tumor (3 to $5 \times 10^7$ cells) were suspended in 3 ml of phosphate-buffered saline. Hypoxia was achieved by passing a stream of water-saturated ultrapure nitrogen over the cell suspension, which was being gently stirred at 37°. After 30 min, 1 ml of degassed NIMP (10 mg) solution was added. A 2nd set of cell suspensions were prepared in air. Four hr after the addition of NIMP, the suspensions were centrifuged at 0° and the pellet in each case was homogenized in 3 ml of distilled water. The homogenates were centrifuged at 40,000 x g for 40 min at 0°. The supernatant was subjected to chromatographic analysis.

**Chemical Reduction of NIMP.** The reduction was carried out with zinc dust in neutral medium in the presence of calcium chloride (14). A solution of NIMP (10 mg in 20 ml of 80% ethanol) was refluxed with 8 mg of zinc dust and 5 mg of CaCl$_2$ for 1 hr. The solution became greenish, gradually turned yellow, and finally became colorless. Completion of the reduction process was confirmed by testing aliquots of the filtrate with a solution of ammoniacal silver nitrate. Hydroxylamine and nitroso derivatives, which are the intermediates in the reduction process, give a gray precipitate with ammoniacal silver nitrate. Absence of a gray precipitate indicated that the reduction to the amine was complete. Upon completion of the reduction, the suspension was filtered and the filtrate was subjected to paper chromatographic analysis as described below. The filtrate did not have an absorbance maximum above 240 nm, confirming the reduction of the nitro group. When a solution of diazotized sulfanilic acid in 10% sodium carbonate was added to an aliquot of the filtrate, the solution turned red. This suggested the presence of an aminomimidazole derivative (20).

**Chromatographic Analysis.** The samples were streaked on Whatman No. 3MM sheets (46 x 57 cm) and were developed for 18 hr using descending chromatography in sec-butyl alcohol saturated with water as the solvent. From the chromatogram, horizontal strips (1 cm wide) were cut out and eluted with water. Aliquots from each eluate were counted for radioactivity.

**RESULTS**

The choice of the incubation conditions for this study was based on the results of previous studies (J. K. Mohindra and A. M. Rauth, personal communication). With 4 hr of incubation at 37°, sufficient amounts of the products were formed. Moreover, negligible amounts of the products were found in the incubation medium. When the cell extracts were prepared as described earlier, 90 to 95% of the total activity associated with cells was found in the water-soluble extracts. If the cell homogenates were treated with trichloroacetic acid, less than 3% of the total activity was associated with the pellet for air-incubated samples while about 12% of the activity was associated with the pellet for nitrogen-incubated cells.

The distribution of radioactivity on paper chromatograms of water-soluble extracts of CHO cells is shown in Chart 2. The results from single-cell suspensions of KHT tumor cells are presented in Chart 3. The patterns for CHO cells and KHT tumor cells incubated in air are apparently similar and show 3 peaks with approximate $R_f$ values of 0.1, 0.4, and 0.6 in addition to that due to NIMP (peak 4, $R_f$ 0.81). The patterns for the extracts of nitrogen-incubated CHO cells and KHT tumor cells are very similar and show the same peaks as the extracts of air-incubated cells with an additional peak with an $R_f$ of 0.2. However, there is a major quantitative difference between the air- and nitrogen-incubated cells. The amount of radioactivity in Peak 2 in nitrogen-incubated cells was about 5 to 8 times more than that of the corresponding air-incubated cells.

As can be seen from Chart 4, the paper chromatogram of NIMP reduced with zinc dust, as described in "Materials and Methods," revealed the presence of only 1 major peak having an $R_f$ value of 0.40, which is the same as that of the major products in the extracts of CHO cells and single-cell suspensions of KHT tumor cells incubated in nitrogen in the presence of NIMP. The identity of the major in vitro cellular product and the chemical reduction product was further identified to that of a sample of RO-07-0582 supplied by Hoffman-LaRoche Inc., Nutley, N. J.
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fibrosarcoma tumor-bearing mouse given an injection of NIMP are shown in Chart 5. The positions of the peaks are apparently similar to those of the cell extracts. The relative radioactivity in the different regions on paper chromatograms of extracts of a number of tissues, plasma, and urine of the mouse are shown in Table 1. The peak with the \( R_f \) of \( \approx 0.1 \) was present on paper chromatograms of urine, plasma, and aqueous extracts of all tissues that have been studied, whereas considerable amounts of radioactivity were also present at the origin on paper chromatograms of extracts of most tissues. The activity in Peak 2 is higher in the tumor than any other tissue except the liver. The activity associated with the pellet is also high in the tumor in com-
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Relative radioactivity in the different peaks of the various organs of a mouse

<table>
<thead>
<tr>
<th>Organs</th>
<th>Relative radioactivity* at peak Rf</th>
<th>% activity in the pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>8</td>
<td>18 69 9</td>
</tr>
<tr>
<td>Kidney</td>
<td>9</td>
<td>2 81 10</td>
</tr>
<tr>
<td>Spleen</td>
<td>8</td>
<td>2 82 11</td>
</tr>
<tr>
<td>Plasma</td>
<td>6</td>
<td>71 10 83</td>
</tr>
<tr>
<td>Urine*</td>
<td>34</td>
<td>3 25 38</td>
</tr>
<tr>
<td>Liver</td>
<td>72</td>
<td>14 2 12 23</td>
</tr>
<tr>
<td>Tumor</td>
<td>19</td>
<td>13 4 64 20</td>
</tr>
</tbody>
</table>

* Expressed as percentage of total radioactivity on the chromatogram.
* Collected over a period of 24 hr.

Comparison with other tissues except the liver. While all of the data on the distribution of radioactivity in the various organs shown in this paper were obtained for tumor-bearing animals, experiments done in non-tumor-bearing animals gave similar results for the distribution of radioactivity in normal tissues.

From paper chromatograms of the urine, sufficient amounts of the materials in Peaks 1 and 3 were obtained for identification studies. The O-demethylation product of NIMP (Chart 6) accounted for the activity in Peak 3. The identification was confirmed by comparing the infrared absorption spectra of the sample isolated from the chromatograms with that of a sample prepared from 2-nitroimidazole according to the procedure of Beaman et al. (5). The glucuronate conjugate of IV and I and of an unidentified product, the spectroscopic properties of which suggest it to be an N1-substituted 2-nitroimidazole, accounted for activity in Peak 1.

DISCUSSION

Based on the data presented above, the major pathways by which NIMP breaks down in the cells are shown in Chart 6. Conjugation with glucuronic acid, O-demethylation, and nitroreduction are the major degradation steps. In the mouse, NIMP is excreted primarily through the urine in the form of free NIMP, the O-dimethylation product, and the glucuronides. The amount of radioactivity per g weight of the different tissues was more or less constant, except for kidney which was approximately 4 times higher. The low percentage of free NIMP present in the liver extracts may be due to the rapid conversion of NIMP to its metabolites.

The formation of relatively high yields of the reduction product in CHO cells and KHT tumor cells incubated with NIMP under hypoxic, but not under aerobic conditions, suggests that hypoxia is a major factor in the conversion of NIMP to the amine by these cells. Thus, the amount of reduction product may be taken as a measure of the extent of hypoxia. As can be seen from Table 1, the amount of the reduction product is highest in the tumor (with the exception of the liver) compared with the other organs of the mouse, suggesting a high degree of hypoxia in the tumor. However, from the present data quantitative estimation of the extent of hypoxia is not possible. Hill et al. (13) have estimated that about 6 to 12% of the cells in a KHT tumor are hypoxic.

It has long been known that the reduction of the nitro group to yield the corresponding amines or hydroxylamines is a major step in the metabolism of aromatic and heterocyclic nitro compounds (12). A number of enzymes capable of catalyzing the reduction of these nitro compounds referred to as nitroreductases are generally active only in the absence of oxygen. The postulated mechanism presumably involves the stepwise reduction of the nitro group to the amine through the nitroso and hydroxylamine intermediates. Generally, the end product depends on the enzyme source.

The increased conversion of NIMP to the corresponding amine by CHO cells and single suspensions of KHT tumor cells under hypoxic conditions is suggestive of the involvement of a nitroreductase enzyme system. A similar enzyme system could explain the presence of the reduction product in the extracts of the different organs of the mouse. The presence of a nitroreductase enzyme system in extracts of many different tissues of a rabbit has been previously demonstrated (11).

One explanation for the higher toxicity of nitro compounds to hypoxic cells (16, 24) is the formation of toxic products. These could be the hydroxylamines, or nitroso compounds, which are probably intermediates in the enzymatic reduction of nitro compounds. The chromatograms of the tumor and liver extracts have comparatively higher amounts of radioactivity near the origin. One probable explanation for this is the binding of reduction products of NIMP to macromolecules. The binding of enzymatic reduction products of nitrofurans to proteins has been previously demonstrated (8, 23). Macromolecular binding of NIMP derivatives could also explain the radioactivity associated with the pellet from the cell homogenates.

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