METABOLISM OF THE CARDIOPROTECTIVE DRUG DEXRAZOXANE AND ONE OF ITS METABOLITES BY ISOLATED RAT MYOCYTES, HEPATOCYTES, AND BLOOD

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ABSTRACT:
The metabolism of the antioxidant cardioprotective agent dexrazoxane (ICRF-187) and one of its one-ring open metabolites to its active metal ion binding form \(N,N\prime\,\text{bis}[N-(2\text{-amino-2-oxoethyl})\text{-glycine}]\) (ADR-925) has been investigated in neonatal rat myocyte and adult rat hepatocyte suspensions, and in human and rat blood and plasma with a view to characterizing their hydrolysis-activation. Dexrazoxane is clinically used to reduce the iron-based oxygen free radical-mediated cardiotoxicity of the anticancer drug doxorubicin. Dexrazoxane may act through its hydrolysis product ADR-925 by removing iron from the iron-doxorubicin complex, or binding free iron, thus preventing oxygen radical formation. Our results indicate that dexrazoxane underwent partial uptake and/or hydrolysis by myocytes. A one-ring open metabolite of dexrazoxane underwent nearly complete dihydroorotase-catalyzed metabolism in a myocyte suspension. Hepatocytes that contain both dihydromorphanidase and dihydroorotate completely hydrolyzed dexrazoxane to ADR-925 and released it into the extracellular medium. Thus, in hepatocytes, the two liver enzymes acted in concert, and sequentially, on dexrazoxane, first to produce the two ring-opened metabolites, and then to produce the metabolite ADR-925. We also showed that the hydrolysis of one of these metabolites was promoted by \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\) in plasma, and thus, further metabolism of these intermediates likely occurs in the plasma after they are released from the liver and kidney. In conclusion, these studies provide a nearly complete description of the metabolism of dexrazoxane by myocytes and hepatocytes to its presumably active form, ADR-925.

Dexrazoxane (ICRF-187, Zinecad; Fig. 1) is clinically used to reduce doxorubicin-induced cardiotoxicity (Hasinoff, 1998; Hasinoff et al., 1998). There is now considerable evidence to indicate that this toxicity may be due to iron-dependent oxygen free radical formation (Malisz and Hasinoff, 1995; Meyers, 1998) on the relatively unproTECTED cardiac muscle. Neutral dexrazoxane, which is permeable to cells (Dawson, 1975), may act through its ring-opened hydrolysis product ADR-925 (Fig. 1), which can either remove iron from the iron-doxorubicin complex (Buss and Hasinoff, 1993) or bind free iron, thus preventing iron-based oxygen radical formation. Thus, dexrazoxane can be considered a prodrug analog of EDTA that is activated upon hydrolysis to its one-ring open intermediates \(\text{B and C}\), and then to its fully ring-opened form, ADR-925, according to the scheme in Fig. 1 (Hasinoff, 1990, 1994a,b, 1998; Hasinoff et al., 1998).

Our previous spectrophotometric and HPLC studies (Hasinoff, 1994a,b) showed that under physiological conditions (37°C and pH 7.4), dexrazoxane is only slowly hydrolyzed to \(\text{B and C}\) \((t_{1/2}\text{ of }9.3\text{ h})\).

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ABBREVIATIONS: ICRF-187, dexrazoxane; ADR-925, \(N,N\prime\,\text{bis}[N-(2\text{-amino-2-oxoethyl})\text{-glycine}]; \text{B, N-(2-amino-2-oxoethyl)-N\prime-(1S)-1-methyl-1,2-ethanediyl]bis[N-(2-amino-2-oxoethyl)]glycine; C, N-(2-amino-2-oxoethyl)-N\prime-(2S)-2-(3,5-dioxo-1-piperazinyl)propyl]glycine; DHPase, dihydromorphanidase and dihydroorotase; DHOase, dihydroorotase; \(\alpha\)-MEM, \(\alpha\)-minimal essential medium; DTPA, diethylenetriaminepentaacetic acid; HPLC, high-pressure liquid chromatography; DRP, DHPase-related protein.
liver, and kidney (Kennedy, 1974), and in erythrocytes and leukocytes (Smith and Baker, 1959), DHPase is only present in the liver and the kidney, but not in the heart (Dudley et al., 1974; Hasinoff et al., 1991; Hamajima et al., 1996). Because dexrazoxane acts as a cardioprotective agent, we investigated the metabolism of dexrazoxane and its one-ring open intermediate C to ADR-925 in neonatal rat myocytes, in adult rat hepatocytes, and in blood with a view to gaining a complete description of its metabolic hydrolysis-activation.

Materials and Methods

Materials. Dexrazoxane hydrochloride and ADR-925 were gifts from Adria Laboratories (Columbus, OH) and were used as supplied. As previously reported (Schroeder et al., 2002), dexrazoxane was assayed by HPLC and was found to contain 0.98, 0.41, and 0.08 mol% B, C, and ADR-925, respectively. HPLC-grade methanol was obtained from Fisher Scientific Ltd. (Nepean, ON, Canada). Tris, Chelex resin, 5-aminoorotic acid, furosemide, L-dihydroorotic acid, and its strongly metal ion-chelating form ADR-925. Where indicated, only DHOase catalyzes the conversion of either B or C into ADR-925.

Results

Metabolism of Dexrazoxane, C, and Dihydroorotic Acid by Myocyte Suspensions. As shown in Fig. 2a, when 10 μM dexrazoxane was added to a myocyte suspension, there was an initial decrease in dexrazoxane concentration in the medium, which was followed by a slower decrease in concentration that paralleled the decrease in concentration in the control experiment in medium alone. A t test comparison of the slopes of all the control data compared with the last four points of the myocyte suspension data (t > 30 min) showed that the limiting slopes were not significantly different (p > 0.2). However, the intercepts were significantly different (p < 0.001), which indicated that the small initial decrease in concentration was statistically significant. An experiment was also conducted in which the loss of 10 μM dexrazoxane from the medium was compared at myocyte cell densities of 2 × 10⁶ and 8 × 10⁶ cells/ml. The decrease in dexrazoxane concentration at 60 min was approximately 3-fold greater at the higher cell density, a result which suggested that some
myocyte, enzymatic, or other process dependent on myocyte density was responsible for the loss of dexrazoxane from the medium. The results of Fig. 2a were suggestive of an enzymatic reaction in which an inhibitory product was produced, halting the reaction. It can be calculated that the 1.8 μM decrease in dexrazoxane concentration from the myocyte suspension of Fig. 2a corresponded to an internal cell dexrazoxane concentration of about 0.4 mM if all of the dexrazoxane was taken up. However, because it has been shown (Dawson, 1975) that razoxane passively diffuses into and out of cells at about the same rate (t1/2 of approximately 20 min), it seems unlikely that myocytes could accumulate this high concentration of dexrazoxane. This calculation is based on our microscopically measured suspended myocyte diameter of 17.2 ± 0.6 μm. Assay sensitivity limitations prevented us from determining whether the neonatal myocytes metabolized dexrazoxane to ADR-925.

As shown in Fig. 2b, when myocytes were treated with C, the myocyte suspension significantly (p < 0.001) increased the rate of loss of C from the medium compared with the control. We previously showed that DHOase enzymatically hydrolyzed B and C to ADR-925, and that 5-aminoorotic acid and furosemide inhibited the hydrolysis of C by purified recombinant DHOase, although 4-chlorobenzenesulfonamide did not (Schroeder et al., 2002). As shown in Fig. 2b, all of these compounds inhibited the loss of C from the myocyte suspension medium. 5-Aminoorotic acid, which is structurally the most similar to C, and furosemide inhibited loss of C by 81 and 92%, respectively, to values that were not significantly different from myocyte control rates (p > 0.2 and p > 0.5, respectively). 4-Chlorobenzenesulfonamide more weakly inhibited the rate of loss of C by 51%, to a rate different from the myocyte control rate (p < 0.01). 5-Aminoorotic acid is a potent inhibitor (Ki, 6 μM) of mammalian DHOase-catalyzed hydrolysis of dihydroorotic acid (Christopherson and Jones, 1980). 4-Chlorobenzenesulfonamide is, however, a noncompetitive inhibitor (Ki, 200 μM) of bacterial dihydroorotase (Pradhan and Sander, 1973) and may inhibit mammalian DHOase as well (Pradhan and Sander, 1973).

To determine whether DHOase was responsible for the loss of C from the myocyte suspension and whether, in fact, myocytes even contained DHOase, experiments were also carried out in which the loss of the DHOase endogenous substrate dihydroorotic acid was determined in the presence of inhibitors (Fig. 2c). As shown in Fig. 2c, the myocyte suspension significantly (p < 0.001) decreased the rate of loss of dihydroorotic acid from the medium compared with the control medium. Pretreatment with 5-aminoorotic acid inhibited the rate of loss of dihydroorotic acid by 88%, to a value that was not significantly different from the control value (p > 0.1). 4-Chlorobenzenesulfonamide and furosemide inhibited loss of dihydroorotic acid by 62 and 41%, respectively, to rates that were different from control rates (p < 0.001 for each, respectively). Together, the results of Fig. 2, b and c, show that myocytes contain DHOase and that the DHOase was, at least in part, responsible for the hydrolysis of C.

Metabolism of Dexrazoxane and C by Hepatocyte Suspensions. We previously showed that adult rat hepatocytes were able to metabolize dexrazoxane to B and C through a DHAPase-catalyzed reaction (Hasinoff et al., 1994). This study showed that metabolites B, C, and D were produced in the dexrazoxane-treated hepatocyte suspension with half-times of approximately 1 h, and then leveled off at 3 to 4 h at a B to C ratio of about 3.5:1. Because the liver contains both DHAPase and DHOase, we decided to investigate whether a hepatocyte suspension could serially hydrolyze dexrazoxane all the way to ADR-925. As shown in Fig. 3a, the concentration of ADR-925 was greatly increased (e.g., 14-fold at 2 h) in the hepatocyte suspension compared with the control experiment. These results indicated that hepatocytes had the capability to completely metabolize dexrazoxane to ADR-925. Also, the appearance of ADR-925 in the medium indicated that ADR-925 effluxed from the hepatocyte after its formation. To rule out the possibility that dexrazoxane hydrolysis was caused by enzymes released into the medium due to a loss in viability of the hepatocytes during the course of the experiment, supernatant medium from a 3-h-old hepatocyte suspension was tested for its ability to hydrolyze dexrazoxane. The rate of dexrazoxane hydrolysis in this medium was not significantly different from the control rate (p > 0.1), indicating that all of the hydrolysis observed in Fig. 3a was intracellular in origin.

To determine whether hepatocytes could metabolize C, and whether DHOase was responsible for the loss of C from the hepatocyte suspension, experiments were carried out in which hepatocytes were treated with C. As shown in Fig. 3b, the hepatocyte suspension significantly (p < 0.001) increased the rate of loss of C from the medium compared with the control medium. As shown in Fig. 3b, 5-aminoorotic acid and 4-chlorobenzenesulfonamide inhibited the
loss of C from the hepatocyte suspension medium by 68 and 52%, respectively, to values that were not significantly different from hepatocyte buffer control rates (p > 0.2). These results indicate that DHOase was, at least in part, responsible for the metabolic hydrolysis of C in the hepatocyte. A comparison of the data of Figs. 2b and 3b shows that the net hydrolysis of C in the hepatocyte suspension was approximately 1.5-fold higher than that in the myocyte suspension. These rates also do not necessarily reflect differing levels of metabolizing enzyme in the two cells, but may reflect differing rates of uptake of C into the cells.

Metabolism of Dexrazoxane and C in Blood and Plasma. We previously showed that dexrazoxane is not hydrolyzed in rat plasma at rates any greater than buffer control rates (Hasinoff and Aoyama, 1999b). We decided to extend these studies to human and rat whole blood and plasma to determine whether they had any dexrazoxane-hydrolyzing activity. The rate of dexrazoxane hydrolysis in human blood and plasma was followed over 4 h (two separate determinations of nine time points; data not shown). The rate of loss of dexrazoxane was no different from that in the Tris/NaCl control buffer (p > 0.5 for each). Similarly, the rates in rat blood and plasma were no different from that in the Tris/NaCl control buffer (p > 0.5 and 0.2, respectively). Thus, it can be concluded that neither blood nor plasma has any dexrazoxane-hydrolyzing activity above that of background control rates (Hasinoff, 1999a,b).

It has been reported that DHOase is present in erythrocytes and leukocytes (Smith and Baker, 1959). Thus, experiments were carried out to investigate whether whole blood and plasma had any C-hydrolyzing activity. We also previously reported that Ca2+ and Mg2+, which are present in plasma at millimolar concentrations, promoted the ring-opening hydrolysis of B and C between 2.5- and 18-fold (Buss and Hasinoff, 1997). The promotion of the hydrolysis of the intermediates B and C likely occurs through the formation of a metal ion complex, as we demonstrated with the Fe3+-B complex (Buss and Hasinoff, 1997). The rate of loss of dihydroorotic acid concentration (which was undetectable) from the medium was not significantly different (p > 0.5) over 90 min in experiments in which 200 µM dihydroorotic acid was incubated with human or rat blood or plasma (data not shown) compared with a control experiment carried out in α-MEM (Fig. 2c). Thus, within the detection limits of our experiments, blood or plasma had no significant DHOase activity.

The results of Fig. 4a and b, showed that relative to a Tris/NaCl buffer control, human and rat blood and plasma all significantly (p < 0.002 and p < 0.001 for rat and human, respectively) promoted the hydrolysis of C by 3.3- to 4.9-fold. To test whether the Ca2+ and Mg2+ present in the plasma were responsible for this increased rate of hydrolysis of C, experiments were carried out in which rat plasma was pretreated with DTPA. DTPA is an extremely strong Ca2+ and Mg2+-binding analog of EDTA (Cheng et al., 1982) and, when present in solution in molar excess of Ca2+ and Mg2+, would reduce the free Ca2+ and Mg2+ to extremely low levels such that, effectively, none would be available to complex the weaker chelator C. Thus, the results of Fig. 5a show that the addition of 5 mM DTPA to rat plasma inhibited the loss of concentration of C from plasma by 97%, to a rate that was not significantly different (p > 0.2) than the Tris/NaCl buffer control rate. The addition of 100 µM DTPA to rat plasma, a concentration which was sufficient to only slightly reduce the Ca2+ and Mg2+ concentration, caused 27% inhibition of the net rate of loss of concentration of C, to a value that just reached statistical significance (p < 0.05) compared with plasma alone. Thus, these results indicated that the Ca2+ and Mg2+ in the plasma were responsible for increasing the rate of hydrolysis of C. The fact that 100 µM DTPA also caused a significant decrease in the rate of loss of C from plasma raises the possibility that there was another component contributing to the loss of C from plasma. DTPA is an extremely strong chelator of the di- and trivalent metal ions Fe2+, Fe3+, Cu2+, and Zn2+ (Cheng et al., 1982), and because we have shown that these metal ions all strongly promote the ring-opening hydrolysis of C (Buss and Hasinoff, 1997), the possibility arises that small amounts of these metal ions, either free or bound to biological components in the plasma, may be promoting hydrolysis of C.

The results of Fig. 5b, in which 5 mM DTPA was added to artificial plasma that contained plasma concentrations of Ca2+ and Mg2+, showed that DTPA significantly (p < 0.001) reduced the rate of hydrolysis compared with artificial plasma. Thus, these results confirm that Ca2+ and Mg2+ make a significant contribution to the hydrolysis of C in plasma.

Discussion

Our previous studies on the metabolism of dexrazoxane in humans (Schroeder et al., 2003) and in the rat (Hasinoff and Aoyama, 1999a;
Schroeder and Hasinoff, 2002) showed that B and C and ADR-925 quickly appeared in the plasma after bolus i.v. administration, a result which suggested that dexrazoxane, and B and C were metabolized. Although dexrazoxane does undergo hydrolysis to ADR-925, this reaction is slow, with dexrazoxane hydrolyzing to B and C with a $t_{1/2}$ of 9.3 h at 37°C and pH 7.4, and to the final hydrolysis product ADR-925 with a $t_{1/2}$ of 23 h (Fig. 1) (Hasinoff, 1994a,b). The fact that the monoanionic B and C and diatomic ADR-925 are present in human and rat plasma at relatively high concentrations (Hasinoff and Aoyama, 1999a; Schroeder and Hasinoff, 2002) also suggested that these three metabolites were being released from the cells in which they were formed, since blood itself does not promote the hydrolysis of dexrazoxane. The metal-chelating ADR-925 (Fig. 1), which can either remove iron from the iron-doxorubicin complex (Buss and Hasinoff, 1993) or strongly bind free iron (Diop et al., 2000), thus preventing iron-based oxygen radical formation, is thought to be the active form of dexrazoxane.

The results of Fig. 2a suggest that dexrazoxane was metabolized by myocytes in a manner that involved product inhibition. The enzyme responsible for this process is unknown but was unlikely to be DHPase since this enzyme is not found in the heart (Dudley et al., 1974; Hasinoff et al., 1991; Hamajima et al., 1996). Homologs of DHPase of unknown function and activity, called DRP-2 and DRP-3 (DHPase-related protein), which have 58 to 59% homology with human DHPase, are found in the heart (Hamajima et al., 1996). Although we did not find dexrazoxane-hydrolyzing activity in the supernatant of porcine heart homogenate (Hasinoff et al., 1991), it is not possible to completely rule out DRP-2 and DRP-3 being responsible for the results of Fig. 2a due to possible inactivation of these enzymes in the homogenate supernatant. The enzyme responsible also cannot be DHOase, since we have shown that it has no dexrazoxane-hydrolyzing activity (Schroeder et al., 2002). The results of Fig. 2a are somewhat consistent with published reports using adult rat heart myocytes, which showed that dexrazoxane is rapidly taken up (Doroshow, 1995) and rapidly metabolized to ADR-925 (Doroshow et al., 1991). Efflux of both dexrazoxane and ADR-925 from myocytes is also equally rapid (Doroshow et al., 1991; Doroshow, 1995). However, we previously showed that dexrazoxane treatment of myocytes only resulted in a slow displacement of iron from an intracellular fluorescence-quenched iron-calcine complex (Hasinoff et al., 2003), a result which suggests that myocytes do not quickly metabolize dexrazoxane to ADR-925. Also, the rapid efflux of dexrazoxane and ADR-925 (Doroshow et al., 1991; Doroshow, 1995) that was seen may have been from a subpopulation of nonviable myocytes, since the efflux of razoxane from BHK-21S cells was not previously reported to be fast ($t_{1/2}$ of approximately 20 min) (Dawson, 1975).

Myocytes, however, do contain DHOase (Kennedy, 1974), and we previously showed that DHOase is able to hydrolyze B and C to ADR-925 (Schroeder et al., 2002). The results of Fig. 2b and c demonstrate that myocytes can effectively hydrolyze both C and the DHOase endogenous substrate dihydroorotic acid, and that this hydrolysis was nearly completely inhibited by the potent and specific DHOase inhibitor 5-aminoorotic acid (Christopherson and Jones, 1980). Although it is possible that these compounds may be blocking cellular uptake rather than inhibiting metabolism, we feel that this is not likely given the diversity in the chemical structures of these...
compounds. These results also demonstrated that C was taken up by myocytes; and, thus, the metabolism of dexrazoxane by DHase in liver cells, and the release of B and C into the plasma where they may be taken up by myocytes, provide a mechanism by which ADR-925 can be formed in the heart and exert its metal-chelating antioxidant protective effects.

The data of Fig. 2, b and c, show that the myocyte suspension increased the rate of hydrolysis of 50 nM C or dihydroorotic acid by about 200 nM/min each. From our previously determined (Schroeder et al., 2002) values of V_{max} and K_m for C and dihydroorotic acid (at 15°C), it is possible to estimate that at 50 µM substrate concentration, DHase in solution would hydrolyze dihydroorotic acid approximately 6-fold faster than C. Thus, the fact that the rates of hydrolysis of the C and dihydroorotic acid in the myocyte suspension were nearly the same suggests that C was taken up by the myocytes more quickly than dihydroorotic acid.

The liver and kidney contain both DHase and DHase (Dudley et al., 1974; Kennedy, 1974; Hasinoff et al., 1991; Hamajima et al., 1996) and thus potentially have the capability to hydrolyze dexrazoxane to its fully hydrolyzed product ADR-925. We previously showed that hepatocytes were able to hydrolyze dexrazoxane to B and C (Hasinoff et al., 1994). The results of Fig. 3a show that hepatocytes were also able to effectively convert dexrazoxane to ADR-925, and that the ADR-925 produced effluxed from the hepatocytes. Hepatocytes were also able to hydrolyze C, and this hydrolysis was largely inhibited by the specific DHase inhibitor 5-aminoorotic acid (Christopherson and Jones, 1980) (Fig. 3b), a result that indicates that DHase was mainly responsible for the metabolism of C in hepatocytes. Although the analysis of the data shown in Fig. 3b indicated that the rates of metabolism of both inhibitors was not significantly different from the control, the rate for both inhibitors was, nonetheless, faster than the control. However, because the intracellular concentration of free Mg^{2+} in hepatocytes is quite high (Gasbarrini et al., 1992) (approximately 0.5 mM, and about one-half that in serum), Mg^{2+}-promoted hydrolysis of B and C (Buss and Hasinoff, 1997) may partially contribute to their metabolism to ADR-925. We also previously showed that myocytes were able to slowly take up B and C and ADR-925 and displace iron from an intracellular fluorescence-quinched iron-calcein complex (Hasinoff et al., 2003).

The results of Fig. 4, a and b, indicated that both blood and plasma promoted the hydrolysis of C. However, even though a previous report had indicated that DHase was present in erythrocytes and leukocytes (Smith and Baker, 1959), we were able to show that neither blood nor plasma was able to significantly hydrolyze dihydroorotic acid. Thus, the increased rate of C hydrolysis seen cannot have any significant contribution from blood or plasma DHase. We previously reported that Ca^{2+} and Mg^{2+} were able to significantly promote hydrolysis of B and C severalfold over its background rate of hydrolysis (Buss and Hasinoff, 1997). The experiments of Fig. 5a in plasma and in artificial plasma containing physiological concentrations of Ca^{2+} and Mg^{2+}, and in which a molar excess of the strong metal chelator DTPA was shown to nearly completely (97%) inhibit hydrolysis of C, indicated that plasma Ca^{2+} and Mg^{2+} were responsible for the promotion of the hydrolysis of C. Thus, these results show that B and C are likely undergoing Ca^{2+}- and Mg^{2+}-promoted metabolism in the circulating plasma. It is not possible to predict whether the metabolism occurring in plasma contributes to or reduces the antioxidant effects of dexrazoxane, since this would depend upon factors such as how long effective circulating levels of B and C are maintained, and the relative rates of uptake of B and C and ADR-925 into the heart tissue, where they could exert their antioxidant effects. However, it should be noted that we were unable to protect myocytes from doxorubicin-induced damage by B or ADR-925 (Hasinoff et al., 2003). Thus, ADR-925 produced in the hepatocyte and released into the plasma may play only a small part in the antioxidant activity of dexrazoxane.

In summary the results of this study have shown that dexrazoxane underwent partial uptake and/or hydrolysis in myocytes by some unknown enzyme or process. The metabolite C of dexrazoxane underwent metabolism in myocytes by DHase. Hepatocytes that contain both DHase and DHase were able to completely hydrolyze dexrazoxane to ADR-925 and released it into the extracellular medium. Thus, these two liver enzymes acted in concert and sequentially on dexrazoxane and, then, B and C to produce ADR-925. These liver enzymes act sequentially because DHase only acts on dexrazoxane, and not B and C, and DHase only acts on B and C, and not dexrazoxane. We also showed that hydrolysis of C was promoted by the Ca^{2+} and Mg^{2+} in plasma, and thus, further metabolism of B and C likely occurs in the plasma after these intermediates are released from the liver and kidney. In conclusion, these studies provide a nearly complete description of the metabolism of dexrazoxane by myocytes and hepatocytes to its presumably active form ADR-925 and how it may be taken up and metabolized to exert its cardioprotective effects in the heart.

References


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