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INVESTIGATION OF THE ROLE OF SERUM LIPOPROTEIN-ASSOCIATED PEROXIDES IN ADRIAMYCIN® CARDIOTOXICITY

RELEASE OF REDUCED GLUTATHIONE FROM RAT HEARTS PERFUSED WITH LIPASE-HYDROLYZED VERY LOW DENSITY LIPOPROTEIN FRACTIONS OBTAINED FROM ADRIAMYCIN®-TREATED AND CONTROL RATS

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Abstract—In a previous study, we demonstrated that the serum of rats treated chronically with the anticancer agent Adriamycin® contains lipid peroxides associated with neutral lipids (W. S. Thayer, *Biochem Pharmacol* **33**: 2259–2263, 1984). In the present study, hearts from untreated control rats were perfused with medium containing serum or very low density lipoprotein (VLDL) fractions obtained from either Adriamycin®-treated rats or control rats. Release of endogenous glutathione from the perfused heart was tested to evaluate possible metabolism of the serum lipid peroxides through the glutathione peroxidase/glutathione reductase redox cycle. Perfusion with lipoprotein lipase-hydrolyzed serum or VLDL caused glutathione release, the extent of which increased with increasing VLDL concentration in the perfusate. The effect was not unique for VLDL from Adriamycin®-treated rats, but instead appeared to be a more general phenomenon since it was also observed with VLDL from control rats. Glutathione was released in the reduced form (GSH), rather than the oxidized form (GSSG) observed during perfusions with model peroxides. Pretreatment of the VLDL with lipoprotein lipase *in vitro* prior to perfusion was necessary in order to obtain GSH release. Neither lipase alone nor palmitate in the absence of lipase was as effective in promoting GSH release. Simultaneous release of lactate dehydrogenase was quantitatively less than that of GSH. The results suggest an action of components of serum VLDL on cardiac membrane permeability. Peroxide metabolism-linked perturbation of the cardiac glutathione redox cycle does not appear to be the mode of action for the serum lipid peroxides found in Adriamycin®-treated rats.

Many *in vitro* studies have documented the feasibility of redox cycling and oxidative stress as a likely mechanism for the chronic cardiotoxicity of the anticancer agent Adriamycin® (doxorubicin hydrochloride)† [1–3]. Formation of oxygen radicals and stimulation of lipid peroxidation *in vitro* have been widely demonstrated with Adriamycin® [4–9]. To investigate the role of such processes *in vivo* [10–13], we have used rats treated chronically with Adriamycin® as an animal model for the heart muscle disease. We have reported previously that such rats show lipid peroxides, or closely related compounds, in their serum [12]. The lipid peroxides were measurable both as thiobarbituric acid reactive material, indicative mainly of endoperoxides, and as hydroperoxides detectable by an iodometric assay. They appeared over a time course similar to that required for the development of cardiac pathology during long-term chronic dosing and persisted after cessation of drug administration. The lipid peroxides were associated with serum lipoproteins, and were found predominantly in the neutral lipid fraction of lipid extracts [12]. In addition, analytical studies of organs indicated that characteristic fluorescent end products of lipid peroxidation reactions were present in the heart and kidney of Adriamycin®-treated rats [13]. These biochemical findings provide evidence for the occurrence of free radical reactions and lipid peroxidation *in vivo* in the rat model of Adriamycin®-induced heart muscle disease [12, 13]. On a molecular level, however, the pathogenetic mechanism linking Adriamycin® redox cycling to cardiac muscle disease remains to be determined.

Cardiomyopathy is associated with chronic, rather than acute, Adriamycin® administration and is both progressive and irreversible [14]. The manifestation of heart disease is often delayed following cessation of Adriamycin® administration and occurs at a time when the drug has been eliminated from the body [14]. These clinical observations suggest that a biochemical mechanism more complex than direct redox cycling of Adriamycin® in the heart is likely involved in the development of cardiac muscle disease. In view of these considerations, we have been investigating the hypothesis that the lipid peroxides found in the serum of Adriamycin®-treated rats may be secondary toxins mediating the development of cardiac muscle disease.

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† Adriamycin is a registered trademark of Farmitalia Carlo Erba.

Studies with model peroxides, such as *tert*-butylhydroperoxide (*t*-BuOOH) and H_2O_2, have shown that these can interact with cellular metabolism by way of the glutathione redox cycle. Peroxide detoxication through glutathione peroxidase can lead to elevation of intracellular oxidized glutathione (GSSG). When rates of metabolism through the peroxidase are high, or when glutathione reductase activity is low, GSSG efflux from cells is observed [15–18]. This process can result in depletion of intracellular glutathione, thus enhancing the susceptibility toward further oxidative stress-related processes. One potential mechanism whereby serum lipid peroxides may act as toxins is through interaction with the glutathione redox cycle. That is, if the serum lipid peroxides were metabolized by cardiac glutathione peroxidase, this could perturb the intracellular glutathione redox state and might lead to depletion of glutathione by GSSG efflux.

To test whether the lipid peroxides found in the serum of Adriamycin®-treated rats might act in a manner similar to that of model peroxides, we have perfused hearts from naive untreated control rats with medium containing serum or very low density lipoprotein (VLDL) fractions from Adriamycin®-treated (and control) rats. Data presented in this paper indicate that such serum can induce small amounts of glutathione release from the heart, provided that the serum triglycerides were hydrolyzed by incubation with lipoprotein lipase prior to the perfusion. However, the characteristics of the process indicate that glutathione release arises by a mechanism different from that of model peroxides. The effects were not due to metabolism of the serum lipid peroxides through the glutathione redox cycle, but instead arose from an alteration of the permeability of cardiac myocyte membranes. The data suggest that the ability to provoke a change in membrane permeability is not unique for lipoprotein components from Adriamycin®-treated rats, but may be a more general phenomenon.

EXPERIMENTAL PROCEDURES

Animals. Male Fischer rats (Charles River, Raleigh, NC) were treated chronically with Adriamycin® hydrochloride (Adria Laboratories, Columbus, OH) at a dose of 1.5 mg/kg/week for 13 weeks by subcutaneous injection as previously described [12]. Blood was collected from neck vessels following decapitation and allowed to clot for preparation of serum. Untreated male Fischer or Sprague–Dawley rats were used as controls for preparation of control VLDL fraction. All rats used for heart perfusion were 300–450 g Sprague–Dawley males.

VLDL isolation. Sera collected from four similarly treated rats (Adriamycin® or control) were pooled and diluted 1:2 with 0.195 M NaCl containing 10 mg/L EDTA, pH 7.5. A combined chylomicron–VLDL fraction was isolated by ultracentrifugal flotation at density = 1.006 g/ml by centrifuging at 50,000 rpm for 17 hr at 14° in a Beckman Ti75 rotor [19]. Fractions were stored at −20° prior to use. These lipoprotein fractions obtained from chronic Adriamycin®-treated and control rats are referred to as ADR-VLDL and control-VLDL, respectively, in the text.

Heart perfusion. Hearts from untreated control rats were excised under Nembutal anesthesia and perfused via the aorta in non-recirculating mode as previously described [18]. The medium was equilibrated with 95% O_2:5% CO_2 with a lung-type oxygenator, and flow rate was maintained at 6 ml/min by a peristaltic pump. The agent to be tested for an effect on the perfused heart (i.e. serum or VLDL fraction) was preincubated in 10 ml of perfusion buffer containing dialyzed bovine serum albumin (20 mg/ml) with or without 1 mg lipoprotein lipase (from *Pseudomonas*, Sigma Chemical Co., St Louis, MO) for 30 min at 37°. This mixture was then added to 220 ml of perfusion buffer, and a valve was switched to allow this solution to flow through the oxygenator and, subsequently, the heart. The perfusion buffer was switched over at 25 min after initiation of heart perfusion, but the apparatus had a dead volume corresponding to about 5 min of perfusion. Thus, the experiments were such that the heart was perfused first for 30 min without the test agent, followed by 30 min with the test agent. Effluent perfusate (0.8-ml aliquots) was collected directly from the heart at 5-min intervals, beginning at 15 min after the start of perfusion and quenched in 0.2 ml of ice-cold 10 mM EDTA or 10 mM EDTA/50 mM *N*-ethylmaleimide (NEM) [15, 16]. At 60 min, hearts were frozen in liquid N_2 and stored at −70°.

For each perfusion, net release was calculated by subtracting the average basal rate of release measured during perfusion times 15–30 min (mean value from four time points) from the average rate of release during perfusion times 32–60 min (mean value from seven points; see Fig. 1). The incremental change in rate of release was then multiplied by 30 to obtain total net release over the 30-min exposure to the test agent. This calculation procedure gave results comparable to estimation of the area under the release versus time curve (AUC) by the trapezoidal method. The percent release was determined from the ratio of total release to the sum of released plus residual tissue amounts for each experiment. Values indicate mean ± SEM.

Biochemical assays. Glutathione in the effluent perfusate and heart was measured spectrophotometrically by the catalytic assay employing glutathione reductase and 5,5′-dithiobis-(2-nitrobenzoic acid) [15, 20]. Total glutathione (GSH + GSSG) was determined from samples without NEM; GSSG was measured in replicate samples quenched with NEM, after extraction with diethyl ether (ten times using 1 ml) to remove excess NEM [15]. GSH was determined by difference. The assay was calibrated in each run by using internal standards of GSSG.

Lactate dehydrogenase (LDH) was assayed in the effluent perfusate and heart homogenates by a spectrophotometric assay [21]. Homogenates were prepared by disrupting 0.1 g heart in 10 ml of 50 mM KP_i/1 mM EDTA, pH 7.4, using a Polytron PT-10 operated at setting No. 6 for 1 min. The homogenate was subsequently sonicated for 10 sec using a microtip of a Branson Sonifier model W-185 at setting No. 4 to ensure complete solubilization of tissue LDH.

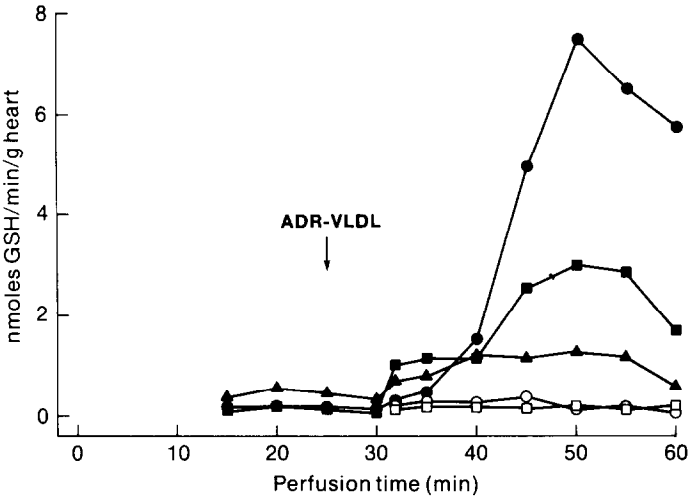

Fig. 1. Requirement for lipoprotein lipase pretreatment of VLDL in order to induce glutathione release from the perfused rat heart. Hearts from untreated control rats were perfused with Krebs–Henseleit buffer containing 5.5 mM glucose and 2.5 mM $CaCl_2$ at 37°. After 25 min, the perfusate was switched to a similar medium containing VLDL obtained from rats that had been treated chronically with Adriamycin® (see Experimental Procedures). The VLDL fraction had been mixed with bovine serum albumin and preincubated for 30 min either with (closed symbols) or without (open symbols) lipoprotein lipase as described in Experimental Procedures. Final concentrations in the perfusion buffer were 0.5% (v/v) ADR-VLDL (equivalent to 0.09 mM triglyceride prior to lipase pretreatment) and 1 mg/ml bovine serum albumin. Glutathione concentration in the effluent perfusate was measured at the indicated times and is expressed as the rate of glutathione release. Data are from a series of experiments conducted with a single preparation of ADR-VLDL and illustrate the range of interanimal variability. Key: (●, ■, ▲) VLDL preincubated with lipase; and (□, □) VLDL without lipase pretreatment.

RESULTS

Glutathione release promoted by serum or VLDL from Adriamycin®-treated rats. We used the perfused heart preparation to test the effects of serum from Adriamycin®-treated rats in an attempt to devise a bioassay for the action of serum factors as possible cardiac toxins. We monitored glutathione release because this has been shown to reflect metabolism of model peroxides [15–18] as well as oxidative stress owing to intracellular peroxide generation [22]. Perfusion of hearts from untreated control rats with medium containing 0.5 to 1.0% serum (or VLDL fraction) obtained from rats that had been treated chronically with Adriamycin® did not itself cause release of glutathione from the heart. However, if the serum fraction was pretreated with commercial lipoprotein lipase to hydrolyze the serum triglycerides prior to perfusion, then glutathione release was observed (Table 1). In control experiments,we confirmed that glutathione release during perfusions with lipase alone in the absence of any serum (or VLDL fractions) was significantly less than when lipase-pretreated lipoprotein fractions were used (Table 1).

A similar pattern of glutathione release was obtained when hearts were perfused with a fraction enriched in VLDL from Adriamycin®-treated rats (Fig. 1). VLDL fraction was tested in these experiments because it is the major triglyceride-rich lipoprotein. In previous studies, we found that the serum lipid peroxides are associated mainly with neutral lipid fractions, particularly triglycerides [12]. In addition, Adriamycin®-treated rats exhibit serum hyperlipidemia [12, 23]. The VLDL, as well as other lipoprotein fractions, are typically elevated 4- to 10-fold in the serum of Adriamycin®-treated rats in comparison to saline-treated control rats [23].

The catalytic assay used for measurement of glutathione is highly sensitive, but does not distinguish directly betweeen GSH and GSSG [15]. Measurements of effluent perfusate samples that had been quenched with NEM present did not exhibit any glutathione, whereas samples quenched in the absence of NEM showed glutathione. These results indicated that the perfusate glutathione was entirely GSH, rather than GSSG. By contrast, release of GSSG is predominant during perfusion of the heart or liver with model peroxides [16–18].

Measurements of the residual GSH and GSSG levels in the heart at the end of the perfusion period showed no change in the intracellular glutathione redox state after perfusion with lipoprotein lipase-incubated serum or VLDL fraction. Mass balance studies, summing the released plus residual glutathione, indicated that initial heart levels corresponded to 1.09 ± 0.12 μmol GSH/g heart and 0.027 ± 0.007 μmol GSSG/g heart, respectively ($N = 23$), in agreement with previously reported values [16].

Time course studies revealed that maximal rates of GSH release occurred about 10–20 min after exposure of the heart to the ADR-VLDL, corresponding to about 40–50 min in the perfusion protocol (Fig. 1). Heart rates were maintained at 120–

Table 1. Glutathione release from control rat hearts perfused with various concentrations of lipoprotein lipase-hydrolyzed serum obtained from Adriamycin®-treated rats*

Serum concentration in perfusate (triglyceride equiv., mM)	Lipase treatment of serum prior to perfusion	Net GSH release/30 min (nmol/g)	(%)	(N)
0 (lipase alone)	(+)	8.9 ± 2.6	0.8 ± 0.3	(5)
0.06	−	4.2	0.4	(2)
	+	24.3 ± 4.6	2.1 ± 0.5	(4)
0.12	−	0	0	(1)
	+	19.4	1.7	(2)
0.22	+	54.9	5.2	(1)
0.43	+	6.9	0.6	(1)

* Relative final amounts of serum in the perfusate are expressed as equivalent concentrations of "triglycerides", as determined by dilution of stock serum having a measured triglyceride concentration. Note that where serum was pretreated with lipoprotein lipase, the perfusate actually contained the indicated amount of "lipase hydrolyzed-triglyceride" rather than intact triglyceride (see text). The protocol for preincubation of serum with lipase is described in Experimental Procedures. Similar preincubations in the absence of serum were used for "lipase alone" perfusions. The final concentration of lipase in the perfusate was 4.3 μg/ml. Bovine serum albumin (1 mg/ml final concentration) was also present in all perfusions. Values are means ± SE where N ≥ 3; other values represent single determinations or the average from two determinations. The number of perfusion experiments (N) is indicated in parentheses.

180 beats/min throughout the hour-long perfusion period. The total extent of GSH release was, however, highly variable between animals (perfused heart preparations).

To enhance the detectability of flux through the glutathione redox cycle, we also tested the effects of pretreating rats *in vivo* with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [24, 25], an inhibitor of glutathione reductase, in some experiments. This was expected to amplify any peroxide-induced shift of glutathione redox state toward GSSG by blocking GSSG reduction [25]. However, BCNU pretreatment neither enhanced glutathione release nor facilitated alteration of the intracellular glutathione redox state in response to perfusion with lipoprotein lipase-hydrolyzed serum or VLDL from Adriamycin®-treated rats. Likewise, *in vivo* pretreatment of rats used for heart perfusion with either aminotriazole [18, 26], an inhibitor of catalase [27], or aminotriazole plus BCNU also did not result in any shift in intracellular glutathione redox state or facilitate GSSG release in response to perfusion with serum fractions from Adriamycin®-treated rats.

Serum specificity for glutathione release. To determine whether glutathione release under these conditions was specific for a serum factor from Adriamycin®-treated rats, we also tested VLDL fraction obtained from control rats. Because untreated control rats have very low triglyceride or VLDL concentrations in their serum, use of the VLDL fraction made it possible to compare ADR-VLDL and control-VLDL fractions over an approximately similar range of triglyceride concentrations. However, it was not feasible to obtain control-VLDL fraction in amounts or concentrations as high as had been obtained from Adriamycin®-treated rats. VLDL from control rats also caused some glutathione release from the perfused rat heart. Again, glutathione release occurred in the form of GSH and was dependent on preincubation of the VLDL fraction with lipoprotein lipase. Analysis of the extent of release as a function of the amount of VLDL added to the perfusion medium suggested that lipase-hydrolyzed VLDL from control rats was similarly effective to that from Adriamycin®-treated rats in promoting GSH efflux (Fig. 2). By contrast to Adriamycin®-treated rats, VLDL from control rats did not show evidence for the presence of lipid-

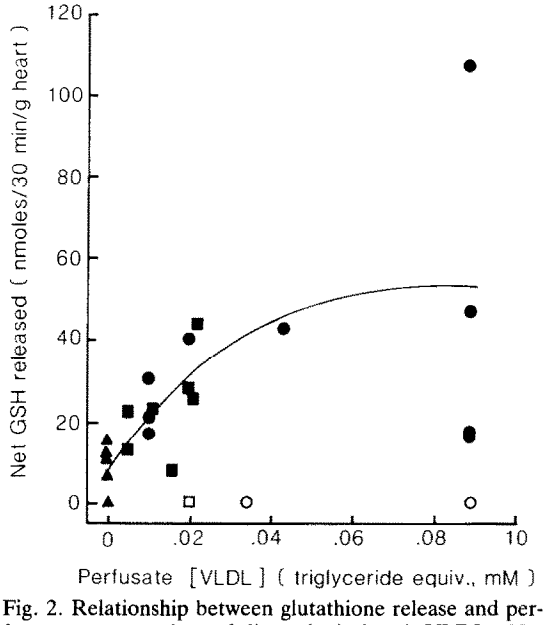

Fig. 2. Relationship between glutathione release and perfusate concentration of lipase-hydrolyzed VLDL. Net glutathione release from control rat hearts was determined as described in Experimental Procedures and Table 1. Key: (●) lipase-treated ADR-VLDL; (■) lipase-treated control-VLDL; (▲) lipase alone without VLDL; (○) ADR-VLDL without lipase pretreatment; and (□) control-VLDL without lipase pretreatment.

Table 2. Effect of chronic Adriamycin® administration on serum and VLDL lipid peroxide and triglyceride levels*

Fraction	Treatment	Thiobarbituric acid reactivity (nEquiv MDA/ml)†	Triglycerides (mM)	(N)
Serum	Control	3.4 ± 0.4	1.0 ± 0.1	(16)
	Adriamycin®	32.1 ± 2.3‡	11.8 ± 0.9‡	(15)
VLDL	Control	1.7 ± 0.8	2.2 ± 0.5	(6)
	Adriamycin®	28.6 ± 8.2‡	10.0 ± 2.9‡	(4)

* Endogenous lipid peroxide content of serum or VLDL fractions used for experiments reported in this study was measured by thiobarbituric acid reactivity as previously described [12]. Triglycerides were measured by a standard colorimetric method [23]. Note that for VLDL fractions, the absolute values per milliliter depend on the dilution made during isolation of the fractions. These dilutions were approximately comparable between preparations from control and Adriamycin®-treated rats. Values are means ± SE; the number (N) of preparations is given in parentheses.

† Malondialdehyde nanoequivalents per ml.

‡ Significantly different from controls at $P < 0.001$ by Student's unpaired *t*-test.

peroxides (Table 2) [12]. In addition, control experiments verified that lipase treatment itself did not induce peroxidation of VLDL. Thus, although our initial rationale for undertaking these experiments was formulated to test the hypothesis that peroxide-type compounds in the serum of Adriamycin®-treated rats might act in the same manner as model peroxides, it appeared from these data that the peroxides *per se* were not responsible for the glutathione release.

The requirement for lipoprotein lipase pretreatment of the serum VLDL fraction suggested that glutathione release required hydrolysis of the serum triglycerides. Thin-layer chromatographic studies verified that lipoprotein lipase had hydrolyzed the majority of triglycerides of serum or VLDL under the incubation conditions employed for the perfusion experiments. Thus, we tested whether palmitate, representative of free fatty acids expected as products of the lipase treatment, would also cause GSH release. (Palmitate was tested without lipoprotein lipase pretreatment.) However, palmitate did not cause significant GSH release above the pre-palmitate basal level of GSH release in four of five experiments (Fig. 3). A fifth experiment showed some GSH release, but this occurred with an aberrant time course compared to the VLDL experiments.

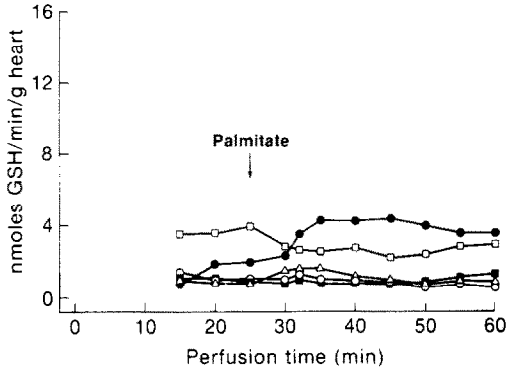

Fig. 3. Effect of perfusion with a fatty acid on glutathione release. Rat hearts were perfused with Na^+/palmitate by a protocol similar to that described for serum or VLDL, but without lipoprotein lipase pretreatment. Dialyzed bovine serum albumin (1 mg/ml) was present throughout all perfusions. Final palmitate concentration in the perfusion medium was 0.03 mM (●, □, △) or 0.30 mM (○, ■).

Comparison of the total glutathione released to the sum of the released plus residual glutathione was used to calculate the percent of the original amount of heart glutathione released by perfusion with the test agent. This was compared with percent release of lactate dehydrogenase as a cytosolic marker for cell disruption. As seen in Fig. 4, GSH release was accompanied by low amounts of LDH release. Quantitatively, however, a significantly smaller percent of heart LDH was released as compared to GSH.

DISCUSSION

Data reported in this paper suggest that components of rat serum VLDL can induce the release of glutathione, and probably other small molecules, from the rat heart. Glutathione release is associated with some agent liberated from VLDL by lipoprotein lipase hydrolysis of the triglycerides. This may not necessarily be a direct triglyceride hydrolysis product such as free fatty acids, however, since palmitate did not produce the same effect. Of course, unsaturated fatty acids may behave differently in this respect. The mechanism of action of the lipase-hydrolyzed VLDL fraction on the heart may involve a lytic, or membrane permeabilizing, activity. This is suggested by the release of lactate dehydrogenase that was also observed. The fact that LDH release was quantitatively less than GSH release on a percent basis suggests that simple rupture of a portion of cells in the perfused heart is not involved, however. A more specific and subtle alteration of membrane permeability which would permit efflux of smaller molecules such as GSH, rather than enzymes such as LDH, is supported by the data.

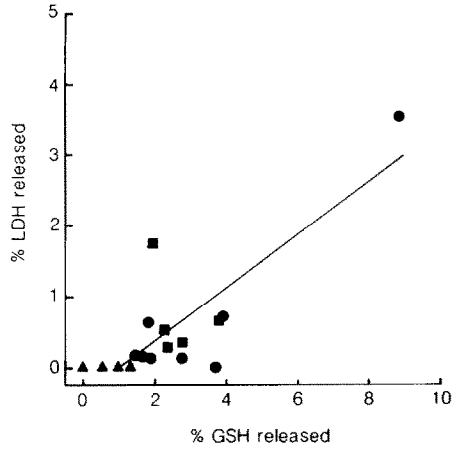

Fig. 4. Relationship between release of glutathione and release of lactate dehydrogenase in hearts perfused with lipoprotein lipase-treated VLDL. Lactate dehydrogenase and glutathione released to the effluent perfusate were measured as described in Experimental Procedures. Residual heart levels of each were also measured at the end of the 60-min perfusion period. Data indicate the net percent of the original amount of each substance released from the heart during perfusion with lipoprotein lipase-treated VLDL. The line was calculated by linear regression analysis; correlation coefficient $r = 0.81$. Key: (●) ADR-VLDL; (■) control-VLDL; and (▲) lipase alone without VLDL.

Our finding that control-VLDL caused GSH release similar to ADR-VLDL, when perfused at comparable triglyceride (VLDL) concentrations, seems to rule out the effect as being caused by an action of peroxide-related compounds. Biochemical characteristics also indicate that the mechanism of glutathione release is not the same as that of model peroxides such as *t*-BuOOH or H_2O_2. Peroxide metabolism does not appear to be involved, since glutathione was recovered in the perfusate as GSH rather than GSSG. This conclusion is further supported by the lack of change in the oxidation–reduction state of glutathione after perfusion with lipase-hydrolyzed VLDL fractions. The absence of perturbations in tissue glutathione redox state or enhancement of glutathione release after rats were pretreated with BCNU, or aminotriazole, or both BCNU and aminotriazole argues against peroxide metabolism through the glutathione redox cycle. These findings are consistent with glutathione release being unreleated to oxidative stress. The presence of peroxide-type compounds in the serum of Adriamycin®-treated rats thus appears not to account for glutathione release. The serum lipid peroxides may, of course, affect cardiac function in a totally different way. Alternatively, these could be end products of the disease process.

The experimental perfused heart system employed for the present study was chosen to permit a close approximation to the heart *in vivo* in terms of route of exposure to serum factors through the vasculature. It is recognized, however, that the perfusion system has an inherent low sensitivity. Thus, the limitations of the system are such that it is not possible to expose the heart to VLDL, or lipid peroxides, at the level actually found in the serum of Adriamycin®-treated rats [23]. Prohibitively large amounts of VLDL would be necessary and would likely lead to complications such as poor oxygen delivery by the perfusion apparatus *in vitro*. Future studies of the effect of lipoprotein components on membrane permeability using cultured cell systems should provide better resolution of the underlying molecular mechanism.

An important consideration is whether a similar membrane-disruptive mechanism could be operable *in vivo* in regard to Adriamycin® cardiotoxicity. In previous studies, we have shown that the activity of heparin-releasable lipoprotein lipase, the active fraction of endogenous enzyme [28], is diminished in hearts of rats treated chronically with Adriamycin® in comparison to saline-treated control rats [23]. Thus, hydrolysis of circulating VLDL triglycerides by the heart might be expected to be low in animals treated chronically with Adriamycin®. On the other hand, the hyperlipidemia which is found in these animals [12, 23] would provide a high concentration of potential substrate for such a hydrolytic mechanism. An action of serum factors as secondary toxins might help explain the delayed and cumulative nature of Adriamycin®-induced heart muscle disease [14]. Further studies will be needed to resolve these points. Nevertheless, the present findings do suggest a new mechanism whereby disturbances of serum lipid metabolism may contribute to Adriamycin®-induced heart disease.

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