Relationship of Cx-reactive Protein Response to Induction of Liver and Kidney Catalases following Hematin Administration into Rabbits

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SUMMARY

The relationship of rabbit Cx-reactive protein (CxRP) and the enzymatic activity of hepatic and kidney catalases in rabbits following i.v. hematin administration has been examined sequentially. The early increases in rabbit liver and kidney catalatic activities were accompanied by the corresponding decreases in β CxRP and an early appearance of γ CxRP after hematin administration. Immunoelectrophoretic analysis of serum samples revealed two types of CxRP: a preexisting, although variable in concentration, β CxRP and a γ CxRP which appeared within 3 hr and then disappeared after 24 hr following a single injection of hematin. Adjuvant-treated rabbits showed slight depression of liver catalase with an increase in β and γ CxRP levels. When hematin was administered to these animals, a rise in both liver and kidney catalases ensued with a concomitant decrease in serum β and γ CxRP's 3 hr after hematin. Actinomycin D, Nogalamycin, and puromycin had essentially no effect on the synthesis of liver catalase in tissue slice culture studies. Purified C-reactive protein interacted with hematin to give variable peroxidatic activity. The data presented suggest the presence of protein precursor pools for the formation of hemoproteins and that, in part, these may be CxRP. Thus, any measure of CxRP or C-reactive protein in pathological situations may be a reflection of changes in the metabolic events of the oxidative system, specifically those of catalases.

INTRODUCTION

The occurrence of common antigens between CRP, HHC, and HEC was demonstrated by Hokama et al. (7) with a variety of immunological procedures. In a more recent report (9), they showed similar physicochemical characteristics between CRP and HHC, which were demonstrated by similarity in disc polyacrylamide and immunoelectrophoretic patterns, and by a pronounced similarity of the tryptic peptide maps of CRP and HHC following 2-dimensional high voltage electrophoresis. In addition, Nishimura et al. (19) showed the occurrence of antigens common to CRP and erythrocyte lysates of acatalasemic individuals when examined with R-AHEC and S-CRPA antisera in agar gel diffusion studies. These workers (7, 9, 19) suggested the possible role of CRP in the formation of hemoproteins such as catalase.

Since the formation of hemoproteins requires both a prosthetic heme group and a protein moiety, it was of interest to determine the effect of the administration of the prosthetic group on the subsequent CxRP response and on the liver and kidney catalase activities in rabbits. An earlier study by Wood and Montella (27) showed that the s.c. administration of homologous CxRP into rabbits stimulated further increase in CxRP 24 hr later in peripheral blood. Purified CxRP added to rabbit liver slices in tissue culture medium showed enhancement of rabbit liver catalase activity (9).

The data reported herein showed that abnormal γ mobility CxRP appeared in peripheral blood of rabbits 3 hr after the i.v. administration of hematin. In addition, induction of the enzyme catalase in the liver and kidney tissues was observed as reflected by the pronounced increase in the levels of the catalatic activity. In essence, this paper presents the data obtained for the CxRP response and liver and kidney catalatic activities following i.v. hematin administration into rabbits. In addition, data for the complex formation of hematin with CRP and its variable effect on the peroxidatic activity of hematin are presented.

MATERIALS AND METHODS

Administration of Hematin into Rabbits. Animals weighing 2.0 to 3.0 kg were given i.v. injections of hematin dissolved in 0.1 M NaHCO₃ buffer, pH 8.4, at a dosage of 0.5 mg/kg body weight. The volume of 0.5% (5 mg/ml) hematin solution administered varied from 0.2 to 0.3 ml. Hematin used in this study was obtained from Calbio-
The enzymatic activity of catalase is reported as the first-nitrogen of the sample examined and thus is expressed previously described (6, 8), from pooled "acute phase" serum were absorbed. In these studies, all the antisera used gel filtration procedures described previously (6, 8). Portions of rabbit liver and kidney were removed for homogenization and examination for catalatic activity.

In 1 experiment, a group of 4 rabbits were given s.c. injections of 3 ml Freund's complete adjuvant prior to i.v. hematin administration at a dose of 0.5 mg/kg body weight. Subsequently, the animals were sacrificed 3 hr after hematin and their CxRP values and catalatic activity of the liver and kidney were compared with untreated and adjuvant-treated rabbits. Six rabbits were treated with 3 ml Freund's complete adjuvant alone and their 24-hr CxRP responses and liver and kidney catalatic activities were examined.

**Immunodiffusion and Immunoelectrophoresis.** The single radial diffusion method of Mancini et al. (14) for the determination of the relative quantity of CxRP in rabbit serum was used. Standard plates containing 10.5% H-CxRPA mixed in 1.5% agarose suspended in 0.1 M citrate-buffered 0.9% NaCl solution, pH 7.2, were used for this evaluation. Each sterile, tightly sealed 5-cm plastic Petri dish contained 2.5 ml antiserum-agarose mixture. Individual rabbit serum was added to a single 3-mm circular well, and the diameter of the radially diffused precipitate was measured with a millimeter ruler to 1.0 ± 0.1 mm after an overnight incubation at 22°. The results were scored as percentage of preinjection values as indicated in Chart 3, or as the diameter measured in millimeters as shown in Chart 4. Immunoelectrophoresis was carried out with microslides with 1.5% agarose suspended in barbital buffer of 0.05 ionic strength, pH 8.6.

**Antisera.** H-CxRPA, S-CxRPA, and S-CRP were obtained from the Hawaii Immunological and Biological Laboratory, Kailua, Hawaii. The antisera were prepared by immunization of horse and sheep with purified γ CxRP and γ CRP obtained by the chromatographic and gel filtration procedures described previously (6, 8). So that the antisera could be rendered specific in some instances, the antisera were absorbed with the appropriate normal sera. In these studies, all the antisera used were absorbed.

**Isolation of CRP.** CRP was isolated by methods previously described (6, 8), from pooled "acute phase" serum samples. It consisted primarily of γ mobility CRP.

**Enzymatic Analysis of Catalase and Peroxidase.** Rabbit liver and kidney homogenates were assayed for catalase activity by the procedure of Beers and Sizer (1). The enzymatic activity of catalase is reported as the first-order reaction constant, K, divided by the mg protein nitrogen of the sample examined and thus is expressed as K/N. The details of the procedure have been reported in previous papers (7, 9).

Peroxidative activity of hematin and hematin-CRP mixtures were determined by the fluorometric procedure of Guilbault et al. (4), with homovanillic acid as the hydrogen donor with H2O2 as substrate. The values of peroxidase are expressed as equivalent units of a known standard of HRP. In some experiments the method described by Maehly and Chance (13), with guaiacol as the hydrogen donor and H2O2 as substrate, was used. The values obtained were expressed as equivalence in μmoles HRP, which was used as the standard.

**Peroxidative Activity of Hematin and Hematin-CRP Complexes.** Varying concentrations of 3 preparations of CRP, S1, S2, S3, were added to hematin and the peroxidative activity was assayed by the guaiacol and fluorometric procedures (4, 13). CRP used in this study was purified and obtained from pool "acute-phase serum" samples. The CRP preparations showed γ electrophoretic mobility in agarose immunoelectrophoresis.

**Liver Tissue Slices in Culture Medium 199.** Normal rabbits were sacrificed by removal of blood via cardiac puncture and the liver was removed aseptically from the abdominal cavity. The liver tissue was rinsed with sterile NaCl solution 3 times and sliced into sections, 3 x 10 x 15 mm. The slices were then transferred to the sterile culture medium contained in 15.0-ml plastic culture bottles.

The medium consisted of 20.0% sterile calf serum in Medium 199 (Hyland Laboratories, Division of Travenol, Los Angeles, Calif.). Nogalamycin (obtained through the courtesy of The UpJohn Company, Kalamazoo, Mich.), varying in concentration from 2.5 to 25.0 μg/ml culture medium, was incorporated into Medium 199. Similar dose ranges were examined with puromycin (Nutritional Biochemical Corporation, Cleveland, Ohio). The level of actinomycin D (obtained through the courtesy of Merck Sharp and Dohme Research Laboratory, Rahway, N. J.) used varied from 25.0 to 250.0 μg/ml culture medium. Liver tissue slices without drugs were carried out in parallel. All preparations were incubated at 37° in 5% CO2 atmosphere. The liver slices were removed at intervals of 6 and 24 hr and homogenized; the homogenates after centrifugation were assayed for catalatic activity. The details of this procedure have been described previously (9).

**RESULTS**

**Effect of Hematin Administration on Rabbit Liver and Kidney Catalases.** The i.v. administration of hematin into rabbits resulted in a rise in catalase activity of liver as early as 1 hr, with the maximum level attained 3 hr after injection. Thereafter, a decline in catalatic activity occurred; however, the levels in some animals remained above the upper limits of the normal values up to 72 hr after hematin administration. These results are summa-
correspond with the radial diffusion analysis; that is, those precipitin bands which were essentially less intense gave smaller radial immunodiffusion precipitin rings. A representative example of this band, which is designated \(\beta\) CxRP for convenience, is shown in Fig. 1c. Fig. 1, b and a, shows the appearance of a second band, designated \(\gamma\) CxRP, which was seen as early as 3 hr (Fig. 1b) and attained its maximum at 24 hr (Fig. 1a) after hematin administration. All animals 1 and 2 hr after hematin was injected showed no \(\gamma\) CxRP in the immunoelectrophoretic examination. Three of the 4 rabbits examined after 3 hr showed the \(\gamma\) CxRP band. Similarly, all the 6- and 12-hr posthematin rabbits injected showed variable levels of \(\gamma\) CxRP. The most intense precipitin bands were noted with all the 24-hr posthematin animals. Reactions of partial identity can be seen in some patterns (Fig. 1a). The 48- and 72-hr rabbits showed no \(\gamma\) CxRP although the \(\beta\) CxRP remained at normal levels. An example of a 48-hr sample run is shown in Fig. 1c.

Results of the examination of the CxRP response following hematin injection by radial immunodiffusion are summarized in Chart 3. The sera examined were the samples from the same animals used for the liver and kidney catalase analysis. The values scored are the percentage decrease or increase of CxRP with reference to the average value from 33 untreated rabbits taken as 100%. This represented a mean value for 33 rabbits of a diameter of 4.4 ± 0.5 mm.

In contrast to the catalytic responses, CxRP showed a corresponding decrease within the first 2 hr following hematin injection. This decrease is due principally to the \(\beta\) CxRP which appears to exist in variable concentrations in untreated animals since at 2 hr no \(\gamma\) CxRP was detectable by immunoelectrophoresis. A gradual rise in CxRP occurs following the minimal decrease after 2 hr.
beginning at 3 hr, until an abnormal increase occurs at 24 hr which subsequently declines to the original levels 48 to 72 hr after hematin injection. This gradual increase results from the appearance of the γ CxRP which attains its maximum level at 24 hr after hematin administration. The sequential appearance of γ CxRP has been discussed in the preceding paragraphs and demonstrated adequately by immunoelectrophoretic patterns,
Chae and Hematin in Catalase Induction

Chart 4. The effect of adjuvant (0 to 24 hr) and adjuvant plus hematin (24 to 27 hr) on the β (O—O) and γ (+——+) CxRP responses.

Fig. 3. Representative immunoelectrophoretic patterns of rabbits treated with adjuvant and later with hematin. a, patterns of Rabbits 173 and 174 before adjuvant or hematin; b, Rabbit 173 24 hr after adjuvant injection and after adjuvant followed by hematin 3 hr after; c, Rabbit 174 24 hr after adjuvant and then adjuvant followed by hematin 3 hr after.

as shown in Fig. 1, a and b. In addition, a representative example of a radial immunodiffusion analysis showing decrease in β CxRP level is shown in Fig. 2a 2 hr after hematin. This is essentially β CxRP since γ CxRP is not detectable at this time after hematin.

Effect of Hematin Administration in Adjuvant-treated Rabbits. Since administration of hematin into rabbits showed an increase in enzymatic activity of liver and kidney catalases, it was of interest to determine the effect of hematin on rabbits with precirculating high levels of γ and β CxRP's. Rabbits given s.c. injections of 3 ml Freund's complete adjuvant were challenged i.v. with 0.5 mg/kg hematin 24 hr after adjuvant treatment. These animals were sacrificed 3 hr after the hematin injection, and the liver and kidney were analyzed for catalatic activity. The serum samples obtained before adjuvant and before and after hematin injection were analyzed for the CxRP responses by radial immunodiffusion and immunoelectrophoresis with H-CxRPA and S-CxRPA.

These results of the liver and kidney catalase activity for untreated, adjuvant-treated, and adjuvant-plus-hematin-treated rabbits are summarized in Tables 1 and 2 for liver and kidney catalase, respectively. Adjuvant-treated rabbits showed a slight decrease in liver catalase activity of approximately 25%. No decrease in activity for kidney catalase was observed for the 6 animals examined at 24 hr. In contrast, both liver and kidney catalase activity showed a marked increase following injection of hematin into adjuvant-treated animals. These are scored in the 3rd column of Tables 1 and 2. Concurrent with this increase in the catalatic activities of liver and kidney was the simultaneous decrease in the γ and β CxRP's from the peripheral serum of 3 out of 4 of these rabbits examined. The quantitative decrease of γ and β CxRP's, as examined by radial immunodiffusion, is shown in Chart 4. The variation of CxRP 24 hr after adjuvant treatment between animals is indeed large. However, the decrease in CxRP 3 hr after hematin is highly significant irrespective of the degree of drop since the optimum CxRP concentration appears generally 48 hr after Freund's incomplete adjuvant. Thus a gradual rise

Table 3
Effect of actinomycin D, Nogalamycin, and puromycin on rabbit liver catalase synthesis in vitro

<table>
<thead>
<tr>
<th>Dose (µg/ml medium)</th>
<th>Catalatic activity after 6- and 24-hr incubation* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D</td>
<td>Nogalamycin</td>
</tr>
<tr>
<td>2.5</td>
<td>115.0</td>
</tr>
<tr>
<td>12.5</td>
<td>102.0</td>
</tr>
<tr>
<td>25.0</td>
<td>83.0</td>
</tr>
<tr>
<td>125.0</td>
<td>105.0</td>
</tr>
<tr>
<td>250.0</td>
<td>116.0</td>
</tr>
</tbody>
</table>

*Control catalatic values at 6 and 24 hr are expressed at 100 ± 15% as determined from 12 separate culture flask of 6 different livers.

Table 4
Peroxidatic activity of hematin and hematin-CRP complexes

<table>
<thead>
<tr>
<th>Sample mixtures</th>
<th>Peroxidatic activity (µmoles HRP = 1 X 10^-5)</th>
<th>Increase in activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5 µg hematin</td>
<td>5.2</td>
<td>83.2</td>
</tr>
<tr>
<td>+ 30 µg CRP S2</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>25.0 g hematin</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>+ 4.67 µg CRP S1</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>+ 13.91 µg CRP S1</td>
<td>11.5</td>
<td>28.0</td>
</tr>
<tr>
<td>+ 13.91 µg CRP S1</td>
<td>11.5</td>
<td>28.0</td>
</tr>
<tr>
<td>+ 15.00 µg CRP S1</td>
<td>11.7</td>
<td>30.0</td>
</tr>
<tr>
<td>+ 27.82 µg CRP S1</td>
<td>11.5</td>
<td>28.0</td>
</tr>
<tr>
<td>+ 55.64 µg CRP S1</td>
<td>12.4</td>
<td>37.9</td>
</tr>
<tr>
<td>+ 55.64 µg CRP S1</td>
<td>15.1</td>
<td>67.9</td>
</tr>
</tbody>
</table>
would be anticipated, especially if hematin acted as a second nonspecific irritant. The significance of the change in CxRP is augmented further by the sequential examination of each individual rabbit. These temporal appearances of CxRP in adjuvant- and hematin-treated rabbits are unlike those for rabbits treated with adjuvant alone as reported previously by Riley et al. (22). Additionally, representative immunoelectrophoretic and immunodiffusion patterns for these changes before and after hematin injection are shown in Figs. 2 and 3. The changes were especially pronounced in the β and γ CxRP regions for Rabbit 174. Following adjuvant injection, both γ and β CRP’s appeared to increase in all but 1 animal. Administration of hematin to adjuvant-treated rabbits was followed by a decrease of varying degrees of both γ and β CxRP’s with 1 exception. In this rabbit, β CxRP showed a decrease following adjuvant administration but increased following hematin injection, whereas the γ CxRP continued to increase slightly following hematin administration.

**Effect of Actinomycin D, Nogalamycin, and Puromycin on Liver Catalase Synthesis in Vitro.** Table 3 summarizes the effect of 3 drugs on the catalase synthesis of liver in vitro. Actinomycin D at concentrations of 25.0 to 250.0 μg/ml culture medium had no effect on the formation of liver catalase at the 6- and 24-hr periods examined. Similarly, the effects of Nogalamycin at concentrations of 2.5 to 25.0 μg/ml culture medium on catalase synthesis in vitro at 6 hr were negligible. Puromycin, a drug which affects the synthesis of proteins, at similar concentration levels to that used for Nogalamycin had no effect on the appearance of catalase activity in the time periods examined.

**Peroxidatic Activity of Hematin and Hematin-CRP Complexes.** Hematin in a solution of 0.1 M phosphate buffer, pH 7.0, showed peroxidatic activity when analyzed with the guaiacol procedure of Maehly and Chance (13) or by the sensitive fluorometric analysis performed with homovanillic acid (4). Addition of purified γ CRP at various concentrations to a constant amount of hematin showed both inhibition and enhancement of the peroxidatic activity. The results for 3 different preparations of γ CRP are shown in Table 4 and Chart 5. The increases in hematin peroxidatic activity analyzed by the guaiacol procedure varied from a low of 28% to a high of 86%. CRP Sγ showed to give the best enhancement of peroxidatic activity at the hematin:CRP ratio of 1:2.5. This study is summarized in Table 4. In another experiment with CRP Sγ and analyzed by the fluorometric procedure, depression of hematin peroxidatic activity was noted at ratios of less than 1:0.5 and 1:1.1. The optimum enhancement was noted at a 1:0.8 hematin:CRP ratio with an increase of approximately 85.1% of peroxidatic activity. These results are shown in Chart 5 for CRP Sγ.

Apparently, different CRP preparations appear to vary in their enhancement of the peroxidatic activity of hematin and this in part appears to be dependent upon random combining ratios or the kind of CRP involved. That CRP complexes with hematin is demonstrated by the change in mobility in agarose of γ CRP. This is shown in Fig. 4 by immunoelectrophoretic analysis with G-CRPA in 1.5% agarose.

**DISCUSSION**

Earlier studies (7, 9, 19) have suggested a possible role for CxRP in the formation of hemoproteins such as catalase. A preliminary observation (9) showed that purified CxRP added to rabbit liver slices in tissue culture medium enhanced catalatic activity. Since the formation of hemoprotein requires both a prosthetic heme group and a protein moiety, the results of administration of the heme moiety into rabbits predictably should prove of great interest. This especially would be of interest with respect to its relationship to the CxRP response. Inductions of liver and kidney catalases did occur following i.v. administration of hematin into rabbits, thus providing support that enhancement of catalase synthesis ensued. The increase in catalatic activity in the liver and kidney occurred as early as 1 hr after hematin with the maximal
activity level attained 2 and 3 hr after injection for kidney and liver catalase, respectively. Subsequently, a decline in activity occurred with a slight increase again at 72 hr after hematin. Attendant with the catalase activity increases were the decrease in β CxRP and the appearance of γ CxRP in the peripheral blood of these rabbits. The optimum level of appearance of γ CxRP coincided with the decrease in β CxRP and the appearance of γ CxRP following administration of hematin is highly compatible with a role for CxRP in the formation of hemoproteins. Compatible with the findings of this study also are the earlier data of Hurlimann et al. (11) and the recent findings of Riley et al. (23) on the site of formation of CxRP. Evidence presented by these workers suggested the liver as site of CxRP formation. The high frequency of occurrences of β CxRP in untreated rabbits is not surprising as high prevalence rates for CxRP in untreated rabbits and CRP in normal individuals have been reported by many investigators (15, 16, 18, 26). However, these were primarily reported by capillary precipitation test, which according to Nilsson (17) is far less sensitive than the radial diffusion procedure. The latter procedure was used in this study. In a study, Nilsson (17) demonstrated that the CxRP showed γ-β mobility in the comparative agar immunoelectrophoresis examination. This appears to be similar to the β CxRP mobility pattern reported herein with agarose gel and horse or sheep CxRPA. Although CxRP with γ mobility was not indicated for rabbits in his study, this fraction appeared to be the significant and consistent abnormal component. That both β and γ CxRP increased following a severe stimulus such as Freund's complete adjuvant was demonstrated in all the rabbits treated in this manner.

Similar examination with radial agar diffusion of a presumably large group of normal individuals showed a high CRP prevalence rate (84% positives) in Swedish men 50 years and older (18). Whether the CRP's were primarily β or γ was not determined. Our recent examinations in immunoelectrophoresis with H-CRPA of groups of patients with gastric carcinoma, tuberculosis, and leprosy showed high frequencies of β CRP in addition to γ CRP. β CRP was also noted in numerous, presumably healthy, individuals. These findings will be reported in detail elsewhere.

Since the administration of adjuvant to rabbits showed a slight decrease in liver catalase activity with appearance of high levels of β and γ CxRP, it was of interest to determine the effect of hematin at this juncture. The mechanisms by which the subsequent decrease in both β and γ CxRP 3 hr after hematin injection with concomitant increase in liver and kidney catalatatic activity occurs may be explained in several ways. A simple likely interpretation is that the elevated levels of CxRP with concurrent increase in hematin (in this case given exogenously) would stimulate the subsequent formation of hemoproteins, and thus would account for the enhanced catalatic activity in the liver and kidney. Alternatively, hematin and/or CxRP could function as inducer for further catalase synthesis. Occurrence of such inducers has been postulated previously by Rechcigl and Heston (20). Additionally, CxRP or CRP might function as dimer precursor molecules of the tetramer apoenzyme moiety in catalase formation. Such a concept is not incompatible with the recent findings regarding biosynthesis of catalase in microbodies compiled in an extensive review by Hruben and Rechcigl (10). Finally, the decrease in CxRP after hematin administration to adjuvant-treated rabbits may also be due to the complexing of CxRP with hematin and the subsequent removal of the resultant complexes by the reticuloendothelial system. However, such complexes were shown to have no catalatic activity in vitro, and hence would not explain the increase in liver and kidney catalatic activities. Nonetheless, that such complexes may occur in vivo with alterations in peroxidatic activity also should be considered. We are now examining such a possibility.

This ability of CRP to complex hematin is reminiscent of the behavior of hemopexin which has been shown to complex with free heme in serum (5, 24). However, that CRP is not hemopexin has been readily demonstrated by negative result in the immunodiffusion analysis with rabbit antihemopexin against purified γ CRP, This affinity of CRP for hematin may in part explain the appearance of CxRP in degenerative muscle tissues in rabbits as demonstrated by Kushner and Kaplan (12), since muscle tissues have high levels of the heme-containing myoglobin.

Attempts to inhibit liver catalase synthesis in tissue culture medium were ineffective with the drugs used. Actinomycin D (21, 25) and Nogalamycin (3), which reportedly affect the DNA to mRNA sequence, had no effect on synthesis of catalase in vitro at least up to 24 hr examination of the liver slices. Likewise, puromycin (2), which affects the synthesis of proteins, showed no inhibition of the catalase synthesis in vitro up to 24 hr of examination. Thus it is highly conceivable that abundant levels of protein precursor and or stable and large pools of mRNA are adequately available for subsequent hemoprotein synthesis at least within the 24 hr period studied. These data are compatible with other findings herein presented and correlate well with previous attempts to inhibit the CxRP response with numerous drugs by Riley et al. (22).

The present data further support our earlier contentions of the possible role of CRP in the formation of hemoproteins and reemphasize again that any measurement of CRP, especially γ CRP, may reflect on the status of the oxidative metabolism associated with hemoproteins, perhaps specifically with that of catalase.

REFERENCES

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