ACCUMULATION OF PROTOPORPHYRIN IX FROM 8-AMINOLEVULINIC ACID IN BOVINE SKIN FIBROBLASTS WITH HEREDITARY ERYTHROPOIETIC PROTOPORPHYRIA
A Gene-Dosage Effect*

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Bovine erythropoietic protoporphyria (EPP) is a recessively inherited disorder of porphyrin metabolism characterized clinically by cutaneous photosensitivity to wavelengths of light in the long ultraviolet range and characterized biochemically by elevated concentrations of free protoporphyrin IX in erythrocytes and feces (1). Ferrochelatase activity is decreased in all bovine tissues examined, e.g., in liver, heart, kidney, marrow, etc. to ~10% of normal activity in affected homozygous cattle, and to 50% in heterozygous cattle that are clinically unaffected (1). The recessive mode of inheritance of EPP in cattle differs from human EPP, in which inheritance is clearly autosomal dominant in nature.

It has been reported that cultured skin fibroblasts (2) or mitogen-stimulated lymphocytes (3) from human patients with EPP accumulate greater amounts of protoporphyrin IX than normal cells when incubated with 8-aminolevulinic acid (ALA), reflecting the inherited deficiency of ferrochelatase activity. In this study, we report that excessive protoporphyrin IX accumulation takes place also in skin fibroblasts from cattle homozygous for the EPP gene defect when cells are incubated with ALA. In addition, skin fibroblasts from cattle heterozygous for the EPP gene defect accumulate intermediate levels of protoporphyrin IX from ALA. These findings indicate that the basic enzymatic defect underlying the bovine EPP disorder, i.e., a deficiency of ferrochelatase activity, can be functionally demonstrated in cultured skin fibroblasts from affected cattle, and that the extent of protoporphyrin IX accumulation is proportional to the dose of the abnormal gene for ferrochelatase activity.

Materials and Methods
Skin Fibroblast Cultures. Fibroblast cultures were established from skin biopsy specimens obtained from six normal animals, three calves homozygous for the EPP gene defect, and four obligatory carriers of EPP. Skin specimens (~30 mm²) were cut into small pieces (1–2 mm²)

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**Abbreviations used in this paper:** ALA, 8-aminolevulinic acid; FBS, fetal bovine serum; Fe, ferric citrate; EPP, erythropoietic protoporphyria.
and 5-8 pieces were placed in a 25-cm² flask in 5 ml of a modified F12 medium (4) supplemented with 20% fetal bovine serum (FBS). Cultures were incubated at 37°C in an atmosphere of humidified air with 5% CO₂. Growth of fibroblasts was obvious by microscopic observation within 1 wk of incubation, and cells were then subcultured every 10 d by a standard technique (5).

**Porphyrin Determination.** The capacity of skin fibroblasts in culture to convert ALA to porphyrins was studied as follows. Fibroblasts grown in a 25-cm² flask were subcultured as described above and 5 × 10⁶ cells were placed into each 1.8-cm² well of a FB16-24TC plate (Linbro Chemical Co. Inc., Hamden, Conn.) with 0.5 ml of the modified F12-20% FBS. Cells proliferated to confluency within 4 d of incubation. After an additional 2 d of incubation in fresh medium, the medium was removed, and the adherent cells were rinsed with serum-free modified F12 medium, and 1 ml of serum-free modified F12 medium supplemented with bovine crystalline insulin (1 μg/ml) was added. ALA (0.6-3 mM) was also added to the growth medium and incubation was then continued for 24 h at 37°C in a mixture of 5% CO₂ and 95% air. In some cultures, CaMg EDTA (2.5-10 mM) and ferric citrate (Fe; 0.03-0.06 mM) were added before this incubation in addition to ALA. After incubation, the medium was removed and the cells were washed once with 1 ml of Earle's buffer. 500 μl of a 1 N perchloric acid: methanol (1:1) mixture was added to cells to extract porphyrins. After 5 min, the perchloric acid:methanol solution was transferred to a 6- × 50-mm glass tube and fluorescence was measured using a microcell holder designed for a Perkin-Elmer MPF fluorometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) as described previously (5). Protein concentrations were determined by the method of Lowry et al. (6) on the perchloric acid:methanol residue after digestion with 200 μl of 0.5 N NaOH at 60°C for 1 h. Crystalline bovine serum albumin was used as standard. Porphyrin formation was expressed as picomoles of protoporphyrin IX/1 mg protein in 24 h. Phenol red was omitted from the culture media and Earle's buffer because it interferes with the porphyrin fluorescence assay. 0.1 M CaMg EDTA was prepared by adding the solution containing stoichiometric amounts of CaCl₂ and MgCl₂ to EDTA solution while maintaining the pH of the mixture between 7 and 8, and was filtered through a 0.45-μ Millipore membrane filter (Millipore Corp., Bedford, Mass.) before use.

**Results**

Skin fibroblasts from cattle that were normal (+/+), heterozygous (+/e), or homozygous (e/e) for the EPP gene defect did not contain detectable porphyrins after incubation in culture with the growth medium alone. However, when incubated with ALA, these cells accumulated significant quantities of porphyrins (Fig. 1). Fibroblasts of all genotypes (+/+, +/e, and e/e) accumulated porphyrins in a dose-dependent manner when ALA was added to the medium. In all instances, fluorescence emission spectra were characteristic of free protoporphyrin IX (7). In contrast to normal human skin fibroblasts that, when incubated with ALA, accumulated 500 pmol protoporphyrin IX/mg protein in 24 h (5), normal bovine skin fibroblasts accumulated only ~50 pmol protoporphyrin IX/mg protein in 24 h under the same conditions. This suggests that the enzymatic steps from ALA to the formation of protoporphyrin IX are less active or, more likely, that ferrochelatase is more active in normal bovine skin fibroblasts than in normal human skin fibroblasts.

In contrast to low levels of protoporphyrin IX observed in normal bovine skin fibroblasts incubated with ALA, skin fibroblasts from e/e cells accumulated a maximum of ~300 pmol protoporphyrin IX/mg protein. Cells from clinically normal parent cows that are obligatory heterozygotes for the EPP gene defect accumulated intermediate levels of protoporphyrin IX in the presence of ALA (Fig. 1).

We have reported earlier that CaMg EDTA is a potent inhibitor of ferrochelatase activity. CaMg EDTA effectively inhibits ferrochelatase action by chelating iron but does not interfere with cell adhesion in a number of cell culture systems, such as skin
fibroblasts (5), mitogen-stimulated lymphocytes (3), and chick embryo hepatocytes (4). In studies of human EPP, we have demonstrated that protoporphyrin IX accumulation is markedly increased by CaMg EDTA in human lymphocytes transformed with mitogens (3), and that the increase caused by CaMg EDTA is almost twofold greater in mitogen-transformed lymphocytes from normal subjects than in cells from patients with EPP. This suggests that about twice as much ferrochelatase is available for inhibition by the chelator in normal mitogen-transformed lymphocytes than in EPP cells.

Effects of CaMg EDTA on protoporphyrin IX formation by bovine skin fibroblasts are shown in Table I. Porphyrin accumulation was stimulated by CaMg EDTA in all three types of cells in a dose-dependent fashion and reached a plateau of 500–550 pmol in 24 h at 5 mM CaMg EDTA or higher. This porphyrin level probably reflects maximal inhibition of ferrochelatase by CaMg EDTA and is similar to maximal values observed in human skin fibroblasts incubated with ALA and CaMg EDTA. The extent of porphyrin accumulation stimulated by CaMg EDTA was ~13-, 5-, and 2-fold in +/+ , +/ε, and ε/ε cells, respectively. These results suggest that significant inhibition of ferrochelatase activity can be brought about by CaMg EDTA, and that the amount of ferrochelatase activity that is inhibitable by the chelator is greatest in the normal cells, least in the homozygous EPP cells, and intermediate in the heterozygous EPP cells.

The effect of adding iron, one of the two substrates of ferrochelatase, on porphyrin accumulation was also examined in cultured bovine skin fibroblasts. Addition of Fe caused significant reduction in protoporphyrin IX accumulation in +/+ and +/ε cells (Table II), suggesting that iron supplementation facilitated the use of protoporphyrin IX for the formation of heme in these cells. The level of protoporphyrin IX in +/ε cells after treatment with iron was in fact brought into the normal range. This finding
Table I

Effect of CaMg EDTA on Porphyrin Formation by Bovine Skin Fibroblasts

| Cells  | Treatment | Protoporphyrin IX, mean ± SEM | Ratio to ALA control of increase in protoporphyrin IX by CaMg EDTA pmoles/mg protein |
|--------|-----------|-------------------------------|----------------------------------------------------------------------------------|
|        |           |                               |                                                                                 |
| +/+    | 1.2 mM ALA | 42.3 ± 10.2                   | —                                                                               |
|        | 1.2 mM ALA ± 2.5 mM CaMg EDTA | 379.8 ± 65.7                   | 9.0                                                                              |
| +/+    | 1.2 mM ALA + 5 mM CaMg EDTA | 553.8 ± 24.6                   | 13.1                                                                             |
|        | 1.2 mM ALA + 10 mM CaMg EDTA | 530.3 ± 49.5                   | 12.5                                                                             |
| +/+    | 1.2 mM ALA | 108.3 ± 4.7                   | —                                                                               |
|        | 1.2 mM ALA + 2.5 mM CaMg EDTA | 352.5 ± 66.7                   | 3.3                                                                              |
| +/+    | 1.2 mM ALA + 5 mM CaMg EDTA | 428.8 ± 83.7                   | 4.0                                                                              |
|        | 1.2 mM ALA + 10 mM CaMg EDTA | 513.0 ± 89.2                   | 4.7                                                                              |
| +/+    | 1.2 mM ALA | 290.2 ± 40.8                   | —                                                                               |
|        | 1.2 mM ALA + 2.5 mM CaMg EDTA | 467.2 ± 75.9                   | 1.6                                                                              |
| e/e    | 1.2 mM ALA + 5 mM CaMg EDTA | 518.0 ± 59.8                   | 1.8                                                                              |
|        | 1.2 mM ALA ± 10 mM CaMg EDTA | 591.8 ± 82.9                   | 2.0                                                                              |

Cell cultures were prepared and treated with chemicals as described in Materials and Methods. Protoporphyrin IX content was determined fluorometrically after incubating cells with chemicals for 24 h.

suggests that there is sufficient ferrochelatase activity in +/+ and +/e cells that can use protoporphyrin IX when excess iron is supplemented. In contrast, the protoporphyrin IX content of e/e cells incubated with ALA was hardly affected by addition of iron, suggesting an almost complete deficiency of ferrochelatase activity in e/e cells.

We also examined the effect of iron on the level of protoporphyrin IX accumulation stimulated by CaMg EDTA. Reduction in the level of protoporphyrin IX formation was observed in all three types of cells when iron was added with CaMg EDTA (Fig. 2). However, the extent of protoporphyrin IX reduction by added iron in cells treated with both ALA and CaMg EDTA was greatest in normal cells, least in EPP cells, and intermediate in heterozygous EPP cells (Fig. 2). Again, the rate of protoporphyrin IX use thus determined probably reflects the level of ferrochelatase activity in these cells.
### Table II

**Effect of Fe on Porphyrin Formation by Bovine Skin Fibroblasts**

| Cells | Treatment | Protoporphyrin IX | Percent disappearance of protoporphyrin IX by Fe |
|-------|-----------|-------------------|-----------------------------------------------|
|       | 1.2 mM ALA | 42.3 ± 10.2       | —                                              |
| 1/1+  | 1.2 mM ALA + 0.03 mM Fe | 24.3 ± 5.0       | 43.6                                          |
| 1/1+  | 1.2 mM ALA + 0.06 mM Fe | 20.6 ± 4.9       | 51.3                                          |
| 1/1-  | 1.2 mM ALA + 0.03 mM Fe | 108.3 ± 4.7      | —                                              |
| 1/1-  | 1.2 mM ALA + 0.06 mM Fe | 33.5 ± 11.3      | 69.1                                          |
| 1/1-  | 1.2 mM ALA + 0.03 mM Fe | 26.2 ± 9.3       | 75.8                                          |
| 1/1+  | 1.2 mM ALA + 0.03 mM Fe | 290.2 ± 40.8     | —                                              |
| 1/1-  | 1.2 mM ALA + 0.06 mM Fe | 300.8 ± 48.0     | 0                                              |
| 1/1-  | 1.2 mM ALA + 0.06 mM Fe | 295.8 ± 53.4     | 0                                              |

Cell cultures were prepared and treated with chemicals as described in Materials and Methods. Protoporphyrin IX content was determined fluorometrically after incubating cells with chemicals for 24 h.

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**Fig. 2.** Effects of iron on the level of protoporphyrin IX accumulated in cultured bovine skin fibroblasts incubated with ALA and CaMg EDTA. Cells were incubated at 37°C for 24 h in a medium containing 1.2 mM ALA and 5 mM CaMg EDTA with or without Fe (30 or 60 µM). Other conditions of the assay were the same as described in the legend to Fig. 1.
Discussion

The results of this study demonstrate a deficiency of ferrochelatase activity in cultured bovine skin fibroblasts with the EPP gene defect. This was achieved by incubating cells with ALA and by fluorometric assay of the amount of protoporphyrin IX formed in these cells. The amount of protoporphyrin IX accumulation appears to reflect the dosage of the abnormal EPP gene.

It has been reported that a radiometric ferrochelatase assay performed in vitro using $^{59}$Fe as the substrate can demonstrate an enzymatic deficiency in cultured human skin fibroblasts from patients with EPP (2, 8, 9). In our hands, this radiometric assay, although suitable for rat liver or chick embryo liver homogenate, is not sufficiently sensitive or reliable for the use in cultured skin fibroblasts nor in lymphocytes. Alternatively, we found that incubation of skin fibroblasts with ALA generates protoporphyrin IX in the cells as a function of ALA concentration in a highly reproducible manner and we have accumulated evidence in a number of systems that this is a reliable reflection of ferrochelatase activity (3, 4). The addition of ALA to cell culture media was originally employed in our laboratory for the detection of uroporphyrinogen-I synthase deficiency in cultured skin fibroblasts (5) or mitogen-stimulated lymphocytes (10) from patients with acute intermittent porphyria (5), and the technique has also been successfully applied by Bloomer et al. (2) to the detection of the EPP gene defect in human skin fibroblasts. Using the same technique, we have also reported the detection of ferrochelatase deficiency in mitogen-stimulated lymphocytes from human patients with EPP (3).

Bonkowsky et al. (8) reported that ferrochelatase activity in skin fibroblasts from human EPP patients is only 10% of that found in normal control cells when enzyme activity was measured by a radiometric method. However, because human EPP is an autosomal dominant disease, patients are heterozygous for a gene defect, such that their ferrochelatase activity would be expected to be 50% of normal. On the basis of the extremely low ferrochelatase activity determined by a radiometric enzyme assay in fibroblasts from human EPP cells, Bloomer recently proposed that ferrochelatase is a multimer whose function can be greatly inactivated with the mutation of one of the subunits of the enzyme (9). However, our studies of bovine EPP clearly indicate that the extent of protoporphyrin IX accumulation is a reflection of ferrochelatase activity, and that this is proportional to the dosage of an abnormal gene in bovine EPP (Fig. 1). A 50% functional deficiency, rather than 90%, of ferrochelatase has been reported from this laboratory in human EPP lymphocytes (3).

Consistent with the deficiency of ferrochelatase activity as assessed functionally by protoporphyrin IX accumulation from added ALA, treatment of bovine cells with CaMg EDTA, a potent inhibitor of ferrochelatase activity, stimulated protoporphyrin IX accumulation in $+/+$, $+/e$, and $e/e$ cells in a decreasing order. This suggests that ferrochelatase activity available for the inhibition by the chelator is greatest in $+/+$ cells and lowest in $e/e$ cells. Similar findings were also obtained in mitogen-transformed human lymphocytes from normal and EPP subjects (3). Our findings also indicate that CaMg EDTA is an efficient inhibitor of ferrochelatase also in cultured bovine skin fibroblasts, as we have shown earlier in cultured human skin fibroblasts (5), mitogen-transformed human lymphocytes (3), and chick embryo hepatocytes (4). CaMg EDTA does not appear to interfere with the Ca$^{2+}$ and Mg$^{2+}$ balance of cells...
BOVINE ERYTHROPOIETIC PROTOPORPHYRIA

because cellular attachment to the substratum, which is dependent on the supply of Ca\(^{2+}\) and Mg\(^{2+}\) (11), is not affected by the chelator treatment.

Another interesting finding in this study is the fact that the treatment of cells with Fe can reduce the level of protoporphyrin IX in bovine skin fibroblasts. Although ferrochelatase is known to use ferrous, but not ferric iron, for incorporation into protoporphyrin IX (12), our finding clearly indicates that sufficient amount of Fe is reduced to ferrous iron, which is then used by the ferrochelatase reaction to form heme. Accumulation of protoporphyrin IX in the presence of CaMg EDTA in +/+ and +/e cells was reduced considerably to less than 200 pmol protoporphyrin IX/mg protein by treatment with Fe, but it remained at 360 pmol/mg protein in e/e cells, a finding consistent with pronounced ferrochelatase deficiency in e/e cells.

In addition to our earlier report on the detection of human patients with EPP disorder using mitogen-stimulated lymphocytes incubated with ALA, the results of our present study of bovine EPP demonstrate the usefulness of measuring protoporphyrin IX accumulation from added ALA for the detection of an EPP gene defect in cultured bovine skin fibroblasts.

Summary

Bovine skin fibroblasts accumulated protoporphyrin IX when incubated in culture with the porphyrin-heme precursor, δ-aminolevulinic acid (ALA). Fibroblasts from cattle homozygous for erythropoietic protoporphyria (EPP) and with the clinical symptoms of the disease accumulated approximately sixfold greater amounts of protoporphyrin IX than cells from normal control animals. Cells from obligatory heterozygous animals, which are clinically normal, accumulated an intermediate level of protoporphyrin IX. When these cells were incubated with ALA and CaMg EDTA, all types of cells accumulated approximately the same amount of protoporphyrin IX (~500 nmol/mg protein), suggesting that ferrochelatase activity was equally low after inhibition by treatment with CaMg EDTA in all cells. Thus the ratio of protoporphyrin IX accumulation from ALA in cultures treated with CaMg EDTA compared with controls treated with ALA alone was greatest in normal cells, least in EPP cells, and intermediate in the heterozygote cells. These findings suggest that the amount of protoporphyrin IX accumulation from ALA reflects the extent of deficiency of ferrochelatase and is proportional to the dosage of abnormal EPP gene in cultured fibroblasts. Similarly, stimulation of porphyrin accumulation by CaMg EDTA reflects diminished ferrochelatase activity in these cells. Thus, the results of this study demonstrate the usefulness of estimating protoporphyrin IX formation from ALA for the detection of an EPP gene defect in cultured bovine skin fibroblasts.

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