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Effects of *in Vitro* Beta-Adrenergic Activation on Rabbit Bone Marrow Erythroid Colony Forming Cells[1] (39801)

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We have previously postulated that the erythropoietic effects of β-adrenergic drugs may be associated with activation of the β-2 adrenergic receptor (1–3). The β-2 receptors may be located in cells in the kidney which produce erythropoietin (Ep). In addition, β-adrenergic receptors may also be located on differentiated red blood cell precursors, the pluripotent stem cell (CFU-S), and/or on Ep-dependent erythroid colony forming cells (CFU-E) (3, 4). Recent reports indicate that salbutamol (albuterol), a long-acting β-2 adrenergic agonist, increases radioactive iron incorporation into newly formed red cells of exhypoxic polycythemic mice (3). These effects were blocked by DL-propranolol, a potent β-1,2 antagonist. It has also been reported that intravenous infusion of salbutamol into conscious rabbits resulted in an increase in plasma levels of Ep (3).

A new sympathomimetic β-receptor-stimulating agent, terbutaline sulfate, has recently been reported to have preferential stimulant action on β-2 adrenoceptors (5). In order to further clarify the possible direct action of β-2 adrenergic activation of the bone marrow erythroid stem cell compartment, the effects of a new β-2 agonist, terbutaline, on CFU-E in rabbit bone marrows were studied. Terbutaline was tested for its influence on *in vitro* heme synthesis in rabbit bone marrow cultures and its *in vitro* effects on the formation of erythroid colonies (CFU-E) in a rabbit bone marrow plasma clot culture system.

Materials and Methods. Effects of terbutaline sulfate and erythropoietin on in vitro heme synthesis. In order to assess the possible direct action of terbutaline on *in vitro* heme synthesis, a modification of the methods of Krantz *et al.* (6) and McDonald *et al.* (7) were utilized. Female New Zealand albino rabbits were sacrificed by cervical dislocation and their femurs were removed. The femurs were cracked open and the bone marrow was removed by flushing it from the femur into a sterile tube with cold NCTC-109 medium (Microbiological Associates). Cell clumps were disrupted in the marrow suspensions by aspirating the suspension several times with a sterile pipet and then washing the cells twice with cold NCTC-109. The final concentration of cells was 1×10^6/ml of culture medium which consisted of 80% NCTC-109 and 20% newborn calf serum. Two milliliters of this suspension were placed in sterile culture dishes (10×35 mm) with either 0.08 U of Ep or terbutaline sulfate in volumes of 20 μl. Three plates were prepared for each group. Both agents were dissolved in NCTC-109 before being added to the culture system. The plates were incubated at 37° in a humidified atmosphere of 95% air and 5% CO_2. After 29 hr of incubation, ^{59}Fe (0.5 μCi/plate) bound to homologous transferrin was added and the incubation was continued for an additional 16 hr. After incubation, the contents of each plate were harvested, the cells were washed twice with cold phosphate-buffered saline, and heme was extracted with cyclohexanone. The data are expressed as percentage ^{59}Fe incorporation into extractable heme in relation to control cultures.

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Plasma clot erythroid colony bone marrow culture. In order to determine the effects of terbutaline sulfate on erythroid colony formation, a modification of the method of McLeod *et al.* (8) was utilized. Female New Zealand rabbits were sacrificed by cervical dislocation and the femurs were removed. The femurs were flushed and washed with collection medium consisting of MEM Hank's Base, nonessential amino acids, sodium pyruvate, L-glutamine, $NaHCO_3$, and heat-inactivated fetal calf serum. The cell concentration was adjusted to 5×10^6 cells/ml. [This suspension (0.1 ml) was added to 0.9 ml of a culture medium composed of beef embryo extract, fetal calf serum, L-asparagine, Ep, NCTC-109, and citrated bovine plasma.] Aliquots of this diluted suspension (0.1 ml) representing 5×10^4 cells with 0.02 U of Ep, terbutaline sulfate, or DL-propranolol per well, separately or in combination, were placed in wells in disposable microtiter plates previously sterilized for 2 hr via uv irradiation. Four clots were prepared for each sample. The microtiter plates were placed in sterile petri dishes containing a water-soaked gauze pad to maintain humidity. The cultures were incubated for 96 hr at 37° in a humidified atmosphere of 95% air and 5% CO_2.

Following incubation, the clots were removed from each well with a spatula and placed on a clean glass slide (four clots/slide). Each clot was blotted dry with filter paper and then fixed for 2 min with 5% glutaraldehyde. Following fixation, the clots were allowed to air dry for 30 min and were stained for 2 min in a 1% benzidine/absolute methanol solution. The slides were then transferred to 2.5% H_2O_2 in 70% ethanol for 2 min, drained, washed, and counterstained with Giemsa solution. A drop of immersion oil was placed on each clot. Each colony composed of cells whose cytoplasm was yellow to orange-brown was scored as a benzidine-positive colony. Benzidine-positive colonies containing eight or more cells were scored under the microscope at 100×.

Results. Heme synthesis in rabbit bone marrow. The effects of terbutaline sulfate and human urinary erythropoietin (0.08 U/plate) on ^{59}Fe incorporation into extractable heme after 45-hr incubation of rabbit bone marrow cultures are presented in Fig. 1. Ep produced a significant increase in percentage ^{59}Fe incorporation into extractable heme (165% of control cultures). Terbutaline alone did not produce a significant increase in ^{59}Fe incorporation into heme in concentrations ranging from 10^{-14} to 10^{-4} *M*

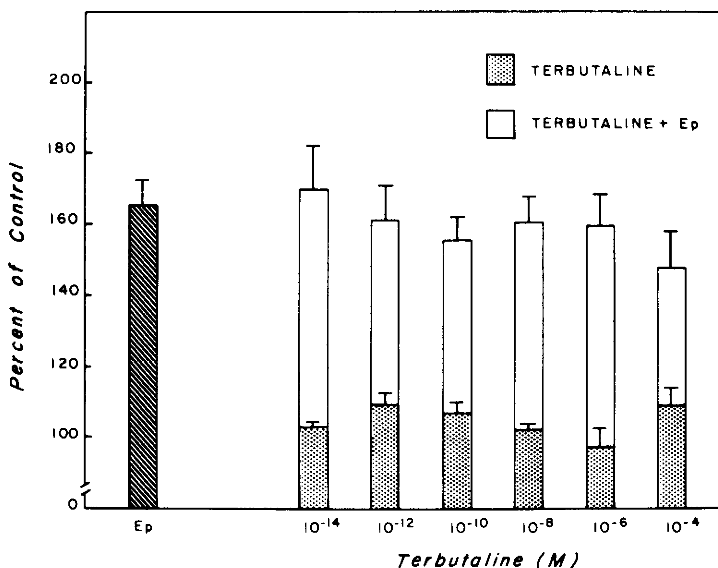

FIG. 1. *In vitro* effects of terbutaline on heme synthesis in rabbit bone marrow cultures. Values are the means ± SE of triplicate determinations from three separate experiments.

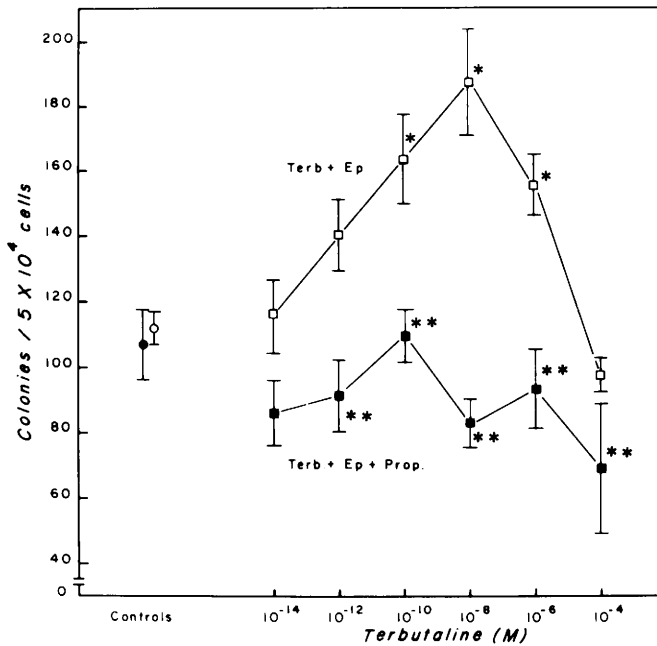

FIG. 2. *In vitro* effects of terbutaline sulfate on the formation of erythroid colonies (CFU-E) in a rabbit bone marrow plasma clot culture system. Values are the means ± SE of quadruplicate determinations from five to seven separate experiments. Controls: ○, Ep (0.02 U/well); ●, Ep (0.02 U/well) + DL-propranolol (10^{-8} *M*). Experimental: □, Ep (0.02 U/well) + terbutaline sulfate; ■, Ep (0.02 U/well + terbutaline sulfate + DL-propranolol (10^{-8} *M*). *Significantly different from controls ($P < 0.05$). **Significantly different from terbutaline + Ep ($P < 0.05$).

when compared with control cultures without drug. In addition, there was no difference in radioiron incorporation into heme of cultures in which both Ep and terbutaline were present over the same concentration range compared to that of Ep alone.

Erythroid colony formation. To further clarify the mechanism of β-adrenergic activation of erythropoiesis, an *in vitro* plasma clot system for erythroid colonies was utilized. The influence of Ep, terbutaline, and DL-propranolol in different concentrations was investigated. Figure 2 shows the effects of Ep and terbutaline sulfate on erythroid colony formation in plasma clots of rabbit bone marrow. Terbutaline in the presence of Ep was found to produce a significant ($P < 0.05$) increase in the number of eythroid colonies at 10^{-10} to 10^{-6} *M* compared to control levels. Terbutaline sulfate was found to be incapable of stimulating erythroid colony formation by itself. When DL-propranolol (10^{-8} *M*) was added to the culture system, it abolished the increase in erythroid colonies seen with terbutaline. No dose of terbutaline sulfate studied in the presence of DL-propranolol was found to produce an increase in erythroid colonies in the bone marrow cultures.

Discussion. Our laboratory has previously demonstrated that the β-2 adrenergic agonist, salbutamol, and the nonspecific β-adrenergic agonist, isoproterenol, were both capable of enhancing erythropoiesis in the exhypoxic polycythemic mouse (1) and the rabbit (3). It is postulated that these agonists stimulate erythropoiesis via β-2 adrenergic receptor activation (1, 3, 9). These drugs probably enhance plasma levels of Ep in experimental animals by exerting a direct metabolic action on the erythropoietin-producing cells of the kidney. Erythropoiesis then proceeds at an accelerated rate due to the action of Ep on the CFU-E in the bone marrow *in vivo*. As postulated previously by Fink and Fisher (1), a direct action of β-2 adrenergic agonists on the bone marrow CFU-E compartment might well be consid-

ered as an additional site of action for this class of sympathomimetic agents. Byron (4) reported that direct exposure of hematopoietic stem cells to isoproterenol caused a marked increase in their sensitivity to the cytocidal action of high-specific-activity [^{3}H]thymidine and that this effect was blocked by DL-propranolol. Byron postulated from these studies that β-2 adrenergic agonist agents may act to trigger hemopoietic stem cells (CFU-S) into cell cycle from G_0 or to shorten their cell-cycle transit time. On the other hand, the Ep receptors which have been postulated by Ortega *et al.* (9) to be on the surface of the erythropoietin-responsive stem cell (ERC or CFU-E) could be affected directly or indirectly by β-2 adrenergic agonists. Singer and Adamson have also reported in preliminary studies that β-2 adrenergic agonists enhance erythroid colony formation in bone marrow cultures in the presence of erythropoietin (10).

Ep was found in the present studies to be necessary for the production of erythroid colonies by adult rabbit bone marrow stem cells in the plasma clot system. These results confirm the observations of McLeod *et al.* (8) and Zanjani (11) with regard to the Ep-dependent nature of erythroid colony formation. Terbutaline, a specific β-2 adrenergic agonist drug, in concentrations between 10^{-10} and 10^{-6} M in combination with Ep produced highly significant increases in the numbers of erythroid colonies in our *in vitro* plasma clot system following 96-hr incubation. It was also found that DL-propranolol would only block the increased number of colonies formed as a consequence of the action of terbutaline. It did not abolish the action of Ep, indicating that only the β-2 receptors were blocked and not those associated with Ep action. Thus it appears that the β-2 agonists potentiate the actions of Ep on erythroid colony formation.

The data in the present study indicate a direct action of terbutaline sulfate on the bone marrow stem cell compartment to increase CFU-E. Our observations also indicate that, in rabbit bone marrow cultures, terbutaline alone or in combination with Ep did not cause an increase in *in vitro* ^{59}Fe incorporation into extractable heme after 45-hr incubation. In addition, terbutaline alone was not capable of triggering hematopoietic stem cells to differentiate and proliferate in the plasma clot system. However, terbutaline in combination with Ep produced an increase in CFU-E which was significantly greater than that of Ep alone. Furthermore, we have observed not only that terbutaline in combination with Ep increased the number of colonies in a dose-related manner, but that some colonies were larger and in a different stage of maturation. The finding of larger colonies following treatment with terbutaline may indicate that this drug is acting on β-2 receptors, possibly triggering cells in the CFU-S compartment into the cell cycle or shortening their cell-cycle transit time, as proposed by Byron (4), resulting in an increase in CFU-E.

Summary. The present study has demonstrated that terbutaline sulfate, a specific β-2 adrenergic agonist drug produces a direct *in vitro* action causing an increase in the number of CFU-E in the rabbit bone marrow cell compartment. Terbutaline in combination with Ep did not cause any greater increase in ^{59}Fe incorporation into extractable heme *in vitro* after 45-hr incubation than Ep alone. However, Ep and terbutaline in concentrations between 10^{-10} and 10^{-6} M produced highly significant increases in the number of rabbit bone marrow erythroid colonies in an *in vitro* plasma clot system following 96-hr incubation compared with Ep alone. Erythroid colony growth in the plasma clot system was found to require Ep. It is postulated that the stimulatory effects of terbutaline on CFU-E are via β-adrenergic receptors, in that erythroid colony formation was blocked by the β-adrenergic antagonist, DL-propranolol. The observation that terbutaline did not produce an increase in heme synthesis may indicate that this β-2 adrenergic agonist is acting on an early stem cell population which is responsive to Ep, in that terbutaline alone was without an effect on the CFU-E cell compartment.

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