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Inhibitory effect of porphyrins on the proliferation of mouse spleen lymphocytes in vitro

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The influence of various porphyrins (deuteroporphyrin IX, mesoporphyrin IX, protoporphyrin IX, hematoporphyrin) and two related compounds (hemin, biliverdin) on the spontaneous proliferation of mouse spleen lymphocytes has been estimated in vitro by the ^{3}H-thymidine uptake assay. It has been found that porphyrins (endogenous ligands for the mitochondrial benzodiazepine receptor) produce a concentration-dependent inhibition of ^{3}H-thymidine incorporation into the DNA of these cells. Metalloporphyrin-hemin has been observed to evoke a weak inhibitory effect, in a high concentration (10^{-4}M), whereas biliverdin, a porphyrins degradation product, was inactive in the same experimental conditions. Those findings indicate that endogenous porphyrins, presumably acting through the mitochondrial benzodiazepine receptor, could regulate the proliferation of mouse spleen lymphocytes in vitro.

Recent evidence suggests that some of the biological actions of many benzodiazepines (BZDs) can be mediated by a peripheral-type receptor found in numerous cells and tissues [1, 2, 3] including the lymphoid tissue [4, 5]. These peripheral benzodiazepine-binding sites are pharmacologically distinct from BZD receptors, located on the neurons, and are not affected by GABA or chloride ions [6, 7]. In addition, subcellular distribution studies indicate that peripheral-type BZD receptor is specifically associated with the outer mitochondrial membrane,

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and being designated as the "mitochondrial benzodiazepine receptor" [8]. Our knowledge of the functional significance for the peripheral BZD receptor in the cellular physiology is still limited. However, several studies have established that the ligands selective for the receptors of this type can induce many effects on nonneuronal cells, including the regulation of the cellular growth and differentiation [9, 10, 11], the neurite outgrowth in PC 12 cells [12], the protooncogene expression [13], and the interference with immune response [14, 15, 16]. It has recently been reported, that many naturally occurring substances and synthetic drugs in addition to benzodiazepines, bind also, with a high affinity, to a peripheral-type BZD receptor [17, 18, 19]. In fact, Verma et al. [20] have shown that various porphyrins bind with submicromolar affinities to the mitochondrial benzodiazepine receptor and proposed that porphyrins may be the endogenous ligand for peripheral-type BZD receptor. In 1987 Snyder et al. [21] postulated that porphyrins acting through peripheral BZD receptor, could be involved in the mitochondrial respiratory control, affecting the cellular proliferation.

In order to check this hypothesis, we have attempted to investigate the effect of various porphyrins and of two related compounds, i.e., hemin and biliverdin, on the proliferation of mouse spleen lymphocytes in vitro. Until now, the role of porphyrins in the regulation of cell growth remains unknown.

MATERIAL and METHODS

Male intact BALB/c mice, approximately 5-6 weeks old, housed in a room with controlled illumination (LD 12:12) and temperature (23 ± 2°C) were used as spleen donors. Mouse spleens were aseptically removed and placed in RPMI-1640 medium (Gibco) with

20 mM Hepes buffer (pH 7.35), penicillin (100 U/ml) and streptomycin (100 ng/ml). Single-cell suspensions from the spleens were prepared at room temperature by gentle teasing of the tissues in RPMI-1640 medium, according to Le Boeuf et al. [22] with our minor modification. The cells were treated by 0.83% ammonium chloride to lyse erythrocytes and washed three times with RPMI-1640. The isolated cells (about 44% T-lymphocytes, 45% of β-lymphocytes and 9% macrophages [23, 24] were checked by trypan dye exclusion method and found to be greater than 95% viable. After 30 min. of preincubation the cells were counted and resuspended in the same medium supplemented with 15% fetal calf serum (Gibco). One ml aliquots of the cell suspension, each containing 10^6 cells were distributed into plastic tubes (12 x 75 mm, Kimble Products, Houston, Texas). The porphyrins, **deuteroporphyrin** (deuteroporphyrin IX-dimethyl ester), **mesoporphyrin** (mesoporphyrin IX-dihydrochloride), **protoporphyrin** [protoporphyrin IX (3, 7, 12, 17-tetramethyl-8, 13-divynyl-2, 18-porphirinedipropionic acid), **hematoporphyrin** (hematoporphyrin-free base), **hemin** (hemin-bovine, type I) and **biliverdin** (biliverdin dihydrochloride) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The investigated compounds, dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Company, St. Louis, MO) and diluted at the desired concentration with RPMI-1640 medium, were added to the appropriate tubes. The final concentration of DMSO was never greater than 0.1% (v/v) and our previous experiments have shown that ^{3}H-thymidine incorporation into mouse spleen lymphocyte DNA was not altered in such conditions. All the tubes were incubated at 37°C in a humidified atmosphere of air CO_2 (95:5%). After 14 hours of incubation, 2 μCi/ml of ^{3}H-thymidine, sp. act. 28.9 Ci/mM (Amersham Centre, England) were added in 100 ml of RPMI-1640 medium. The incubation

was terminated 24 hours later and the cells were washed with 2 ml cold 0.9% NaCl. The replication of DNA and the determination of radioactivity ([^{3}H] cpm/10^6 cells) were conducted, according to previously published methods [25]. The incorporation of ^{3}H-thymidine into lymphocytes, as determined by this procedure, was shown to be a correlate of cell growth [26]. The inhibition of proliferation was determined from the difference between the treated and control cell-associated ^{3}H-radioactivity.

EC_{50} (the half effective concentration) expressed the antiproliferative potency and was determined as follows: control radioactivity was used as 100% and all other radioactivity values were expressed as % of the control. EC_{50} was calculated as the concentration required to reduce ^{3}H-thymidine uptake to 50% of the control. Student's t-test was used to determine the statistical significance.

RESULTS

The data concerning the effect of dicarboxylic porphyrins, hemin and biliverdin on the incorporation of ^{3}H-thymidine into DNA of mouse spleen lymphocytes are given in Figure 1. When cells were cultured for 24 hours in the presence of deutero-, meso-, hemato-, and protoporphyrin the proliferative response of spleen cells was inhibited in a dose-dependent manner, up to submicromolar concentration. A maximal reduction of ^{3}H-thymidine uptake was observed at 10^{-4}M compounds concentrations. The half-effective concentrations (EC_{50}) for inhibition are shown in Table 1. In our assay system deuteroporphyrin IX revealed the highest potency for inhibition and suppressed the lymphocyte proliferation at the lower concentration (10^{-6}M), in comparison to other porphyrins (10^{-5}M). Hemin significantly suppressed lymphocyte proliferation only in the highest tested concentration

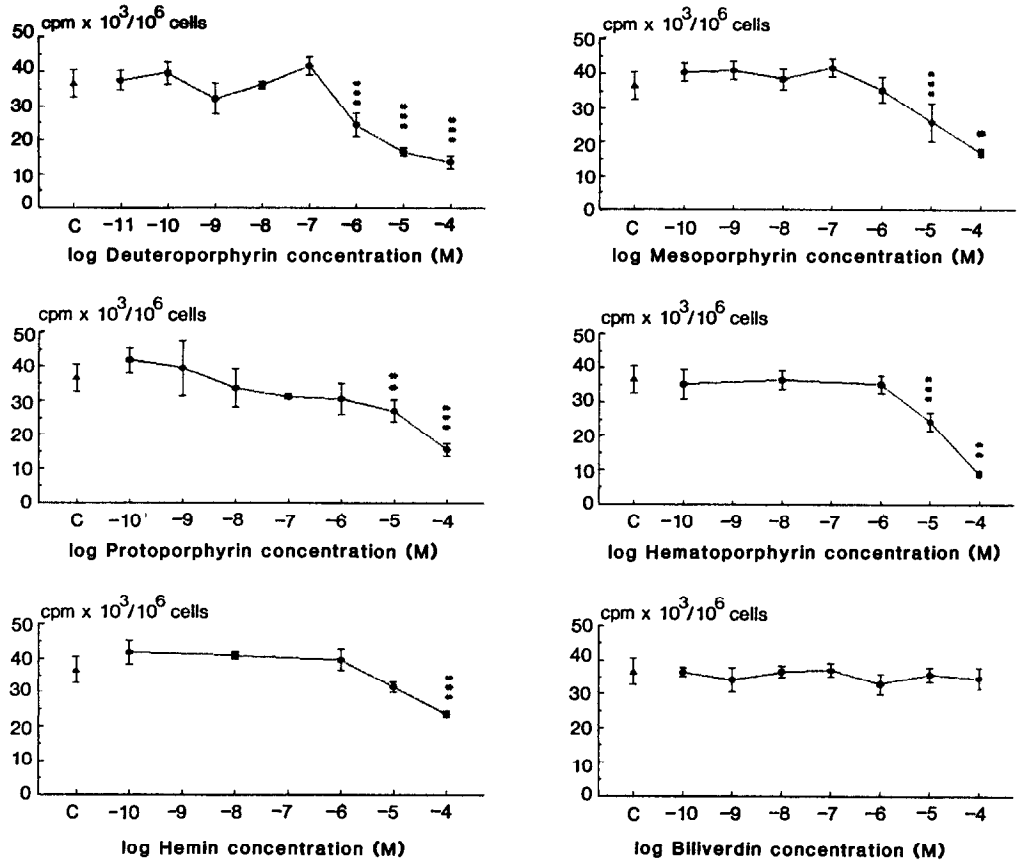

Figure 1.

Inhibition of ^{3}H-thymidine incorporation into DNA of spleen lymphocytes by porphyrins. ^{3}H radioactivity represents the mean ± SE cpm of ^{3}H-thymidine incorporated for three replicate cultures.

*p<0.05, **p<0.01, ***p<0.001, v.s. Control

(10^{-4}M). On the other hand, biliverdin did not exert any significant changes in thymidine incorporation into DNA of spleen lymphocytes.

DISCUSSION

The results of the present experiments clearly indicate that dicarboxylic acid porphyrins, which have been characterized as active ligands of the peripheral type BZD receptor, elicit a profound suppression of the cellular proliferation, manifested as

Table 1

The antiproliferative potencies of porphyrins on mouse spleen lymphocytes

Compound	Range of concentration (M)	EC_{50} (M)
Deuteroporphyrin IX	$10^{-4} - 10^{-11}$	9.24×10^{-6}
Mesoporphyrin IX	$10^{-4} - 10^{-10}$	9.41×10^{-5}
Protoporphyrin IX	$10^{-4} - 10^{-10}$	8.73×10^{-5}
Hematoporphyrin	$10^{-4} - 10^{-10}$	6.60×10^{-5}
Hemin	$10^{-4} - 10^{-10}$	$> 10^{-4}$
Biliverdin	$10^{-4} - 10^{-10}$	$> 10^{-4}$

an inhibition of the incorporation of ^{3}H-thymidine into mouse lymphocyte DNA. On the other hand, hemin and biliverdin, which exhibited lower affinity for mitochondrial type BZD receptor, were weak or inactive in our assay system. All the tested agents were not cytotoxic, for, even after 24 hours of incubation in the presence of compounds more than 95% of spleen lymphocytes remained intact, as judged from the exclusion of trypan blue solution. That suggests that the occupation of peripheral-type benzodiazepine receptors by porphyrins results in a reduction of ^{3}H-thymidine incorporation and lymphocyte DNA replication. These data are consistent with the observation of Wang et al. [27], who found a strong correlation between the antiproliferative actions of a series of BZDs (EC_{50}) and their binding constants for the peripheral-type BZD sites. Further support for this hypothesis is provided by our studies concerning the inhibitory effect of peripheral and mixed type BZD, but not central type ligands on the proliferation of mouse spleen lymphocytes [28, 29] and human glioma cells cultured in vitro [30]. The antiproliferative

activity appeared also to be mediated through the binding of BZD to their specific, high affinity peripheral receptor, located on the cells. Thus, all of these data are consistent with the assumption that peripheral-type benzodiazepine receptor may be involved in the inhibitory effect of porphyrins on lymphocyte proliferation.

The molecular mechanism by which porphyrins inhibit DNA synthesis and lymphocyte proliferation are not fully understood at this time. However, the involvement has been proposed of mitochondrial outer membrane protein called porin [21, 31], which functions as a modulator of the cellular respiration and oxidative phosphorylation, in association with the peripheral BZD receptor activation [31, 32]. Furthermore, the ligands for the peripheral-type BZD receptor have been claimed to interact with the voltage-dependent calcium channels [33, 34], and displaying a micromolar affinity for the dihydropyridine binding sites [35]. Ca^{+2} seems to be an essential modulator of a large number of cellular processes, including the inhibition of DNA synthesis and cell division [36]. In this respect, it is interesting that benzodiazepines, including Ro5-4864, diazepam and PK 11195 at concentrations of 10^{-6} - 10^{-4}M, inhibited plasma membrane Ca^{+2} influx [37, 38] and decreased the duration of Ca-dependent spikes recorded in embryonic dorsal root ganglion neurons grown in tissue culture [39]. The antiproliferative actions of some porphyrins occur at concentrations similar to those required to occupy these micromolar sites, and this would suggest that these sites are involved in the antiproliferative effects. Further studies will be necessary to elucidate porphyrins interaction with intracellular signal systems involved in the regulation lymphoid cell proliferation.

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