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SHORT COMMUNICATIONS

Diphenylhydantoin induction of intracellular β-glucuronidase and alkaline phosphate activity in cultured bovine dental pulp cells

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Diphenylhydantoin (DPH) or phenytoin (previously known as Dilantin; Parke, Davis & Co., Detroit, MI) is the 'drug of choice' in the treatment of grande-mal epileptic seizures. Patients are often treated with the drug for many years and may develop side-effects of varying clinical severity and importance.

One of these side-effects is the induction of numerous enzymes, particularly those found in the microsomal fraction of the liver. Among those thus far described are microsomal enzymes and lactic dehydrogenase in the liver [1] and oxidative enzymes (cytochrome oxidase, NADH cytochrome *c* reductase, and NADPH cytochrome *c* reductase) in gingiva [2]. DPH has been shown to interfere with proline hydroxylation [3, 4] and also appears to alter hydroxylation of vitamin D to that of the more polar inactive metabolites [5, 6]. Many other effects related to abnormal collagen metabolism have been described [7–9].

During a pilot study of bovine dental pulp cells grown in monolayer culture when DPH was included in the medium, we noticed a great increase in intracellular β-glucuronidase, demonstrated histochemically, in these cells as compared to controls but could show no corresponding biochemical phenomenon [10]. We report in this paper a more detailed study which explains the initial discrepancy.

METHODS AND MATERIALS

(A) *Cells*

Bovine dental pulp cells were prepared by ficin dissociation according to the method of Miller *et al.* [11].

(B) *Cultures*

Cells from (A) were counted and appropriately diluted in culture medium to give 10^6 viable cells/25 cm^2 Falcon plastic T-flask. These were cultured in Minimal Essential Medium (MEM) with Hanks' salts and 10% (v/v) fetal calf serum. The medium contained DPH [27 μg/ml (10^{-4} M); 2.7 μg/ml (10^{-5} M); or 0.27 μg/ml (10^{-6} M)] and ethanol (1%, v/v), ethanol (15%, v/v) alone, or no additives. Ethanol was used to initially solubilize DPH.

(C) *Cell sonicates*

(1) Other cells from (A) were further washed with physiological saline, centrifuged, and resuspended in 5 ml saline. The suspension was immersed in an ice bath and sonicated with a Branson (W185 Heat Systems, Ultrasonics, Inc., Plainville, NY) sonicator for 2 min.

(2) Cultured cell sonicates were prepared by decanting medium and cutting off the cell-free side of each Falcon flask with the flattened tip of a soldering iron. (Cells were not allowed to dry.) The monolayer was washed with 2 ml of physiological saline, which was decanted, and another ml of saline was added. Cells were scraped free with a rubber scraper and the saline–cell suspension was carefully removed with a pipette. An additional 1 ml of saline was added to the flask, which was then agitated, and this rinse was added to the cell suspension. The suspensions were stored on ice and sonicated as above.

(D) *Histochemical methods on monolayer cultures* in situ

(1) *β-Glucuronidase* [12]. Naphthol AS-BI glucuronide substrate was incubated with hexazotized pararosaniline in acetate buffer at pH 5.2. Substrate concentration was double the amount recommended by Pearse [12] because higher substrate concentrations are necessary to penetrate the semi-intact cell membranes of monolayers. A control was run which contained only diazonium salt and no substrate.

(2) *Alkaline phosphatase* [13]. The simultaneous azo-coupling method of Gomori [14], which utilizes α-naphthol acid phosphate as a substrate and fast blue RR as a diazonium salt, was employed. Substrate concentration was four times the suggested amount. Again, substrate-free controls were included.

(E) *Biochemical methods on cell sonicates*

All biochemical methods employed were micro-modifications of Sigma kit methods (Sigma Chemical Co., St. Louis, MO) except that for total protein [15] which was also modified to a micro-method. Details of these modifications are given with each technique. Results are expressed as arbitrary units because of these modifications, but comparisons are made only within experiments. No attempt has been made to convert these data to the usual clinical units. All methods are colorimetric and were read on a Bausch & Lomb colorimeter (Spectronic 20) with a microcuvette adaptor and matched microcuvettes.

(1) *pH Profile of β-glucuronidase in uncultured dental pulp cells.* Cell sonicates prepared as in (C1) were assayed for β-glucuronidase [16] by combining 50 μl sonicate with 50 μl phenolphthalein-glucuronic acid (0.03 M) and 200 μl of 0.2 M sodium acetate–acetic acid buffer at pH 4.5, 4.75, 5.0, 5.25 and 5.5 in 2-ml test tubes. These were stoppered, agitated, and incubated at 56° in a water bath for 1 hr. At the end of the incubation, 1 ml of 0.1 M 2-amino-methyl-1-propanol buffer, pH 11, was added. This method was modified from a Sigma kit (Sigma Technical Bulletin No. 325). Absorption was read at 550 nm against a reagent blank. Results were compared with a phenophthalein calibration curve and expressed as μg/ml phenophthalein × 10^{-3}. Each pH point was assayed in triplicate and the results presented are an average.

(2) *Comparison of β-glucuronidase activity at pH* 4.5 *and* 5.25 *in cells cultured with* 0.27, 2.7 *and* 27 *μg/ml DPH.* Sonicates were prepared as in (C2) from three flasks from each experimental group and duplicate assays were performed on each sonicate. Reagents were identical to those in (E1) but proportions were 100 μl cell sonicate to 50 μl substrate and 300 μl buffer at pH 4.5 or 5.25. One milliliter of 0.1 M 2-amino-methyl-1-proponal buffer was added to the tubes after incubation.

(3) *Alkaline phosphate activity in cells cultured with* 0.27, 2.7 *and* 27 *μg/ml DPH.* Cell sonicate (50 μl) was added to 100 μl *p*-nitrophenyl phosphate substrate [100 mg Sigma 104 phosphatase substrate (Sigma Technical Bulletin No. 104) to 33.2 ml water] and 250 μl of 0.1 M glycine in 0.001 M magnesium chloride, pH 10.5. Tubes were stoppered, agi-

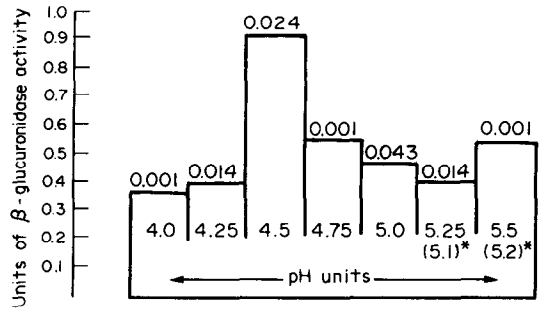

Fig. 1. β-Glucuronidase activity in bovine dental pulp at a range of pH values. Notice that the prominent peak at pH 4.5 is quite distinct from the lesser peak at pH 5.2. The number at the top of each column represents the standard error. Values indicated by an asterisk (*) show pH at the end of the assay if a change occurred.

tated and incubated for 30 min at 37°. One milliliter of 0.2 N NaOH was added, and absorbance was read at 420 nm and compared with a calibration curve of *p*-nitrophenol. Duplicate assays were made on each of the three sonicates from each group.

(4) *Total protein.* Total protein was determined by a micro-modification of the method of Lowry *et al.* [15]: 100 μl cell suspension was added to 1 ml of Lowry's solution C and 50 μl of Folin and Ciocalteu's reagent diluted 1:1 with distilled water. Tubes were incubated for 1 hr at room temperature. A calibration curve was prepared with dilutions of Sigma protein standard (Sigma Stock No. 540–10:50 mg/ml human albumin, Fraction 4, and 30 mg/ml human globulin, Fraction II) incubated at the same time. The results were read against this and expressed as μg of protein/ml. Each sonicate was assayed in triplicate. The results from biochemical methods (E2) and (E3) were corrected for total protein.

RESULTS

Figure 1 and Table 1 summarize the data obtained from the biochemical studies.

β-Glucuronidase activity in uncultured bovine dental pulp cells maximized sharply as the pH was increased to 4.5 but decreased as the pH became more alkaline (Fig. 1). At pH 5.5 activity again increased. The minimum at pH 5.25 is not statistically significant but the increase at 4.5 is highly significant. A subsequent pH check showed that the pH shifted slightly downward during the reaction.

β-Glucuronidase activity in cultured cells, when assayed at pH 4.5, reflected no significant differences between cells cultured with ethanol or any concentration of DPH (Table 1). When the assay was carried out at pH 5.2, however, a very different picture was obtained (Table 1). The ethanol control did not differ from the medium control, but addition of DPH in even the lowest concentration (0.27 μg/ml) produced a very significant increase in biochemically demonstrable β-glucuronidase. The next concentration (2.7 μg/ml) produced yet another statistically significant increment, but a further increase in DPH concentration to 27 μg/ml did not elicit any greater β-glucuronidase activity.

The per cent activity at pH 5.2, when compared to that at pH 4.5, was increased from approximately 60 per cent in the controls to 85–90 per cent in DPH-treated cultures.

Histochemical analysis of β-glucuronidase, at pH 5.2, in contrast to the biochemical findings, demonstrated a consistent and corresponding increase in enzyme activity with increasing DPH concentration in the culture medium. The increase in activity between 2.7 and 27 μg/ml DPH was very marked, however, while the increase between controls and 0.27 and 2.7 μg/ml DPH, while detectable, was not so striking.

Biochemically demonstrable alkaline phosphatase was found to increase only when cells were cultured with 27 μg/ml DPH (Table 1). The increase was significantly different from the medium control but not from the ethanol control, most probably because of the large standard error of the latter. The depression of activity at 0.27 μg/ml is not statistically significant. Histochemically, no increase in alkaline phosphatase activity could be discerned readily.

DISCUSSION

The pH profile of β-glucuronidase present in uncultured pulp cells suggests that two isoenzymes may be present. Mills [17] has shown that isoenzymes exist which have maxima of 4.5 and 5.2. The maximum at 4.5 is obvious (Fig. 1) but the maximum at the more alkaline pH is not

Table 1. β-Glucuronidase and alkaline phosphatase activity of cells cultured with and without diphenylhydantoin

Culture conditions	β-Glucuronidase, pH 4.5 [(units × 10^{-3}/μg protein) ± S.E.]	β-Glucuronidase, pH 5.2 [(units × 10^{-3}/μg protein) ± S.E.]	Alkaline phosphatase [(units × 10^{-3}/μg protein) ± S.E.]
Medium control (MEM)*	1.82 ± 0.31	1.03 ± 0.12	4.92 ± 0.33
Ethanol control (MEM + 1% EtOH)†	2.04 ± 0.44	1.24 ± 0.25	4.88 ± 0.69
0.27 μg DPH/ml† (10^{-6} M)	2.77 ± 0.13	1.94 ± 0.07 ($P < 0.025$)‡	4.39 ± 0.42
2.7 μg DPH/ml† (10^{-5} M)	2.69 ± 0.26	2.43 ± 0.21 ($P < 0.0005$)‡	5.62 ± 0.48
27 μg DPH/ml†, § (10^{-4} ppM)	2.97 ± 0.33	2.67 ± 0.27 ($P < 0.005$)‡	6.07 ± 0.20 ($P < 0.05$)‖

* Minimal essential medium, Hanks' salts, 10% calf serum.

† Diphenylhydantoin was obtained from Parke Davis & Co. and added to the medium as a 100 × conc in ethyl alcohol.

§ The normal plasma concentration of this drug is 7–30 μg/ml.

‡ Significance of difference from EtOH control.

‖ Significance of difference from medium control.

quite as striking. The apparent slight shift in the 5.2 max is due to the pH having dropped during the assay, and later assays on cultured pulp cells contained a larger proportion of buffer in an attempt to alleviate this problem.

The statistical analysis on β-glucuronidase assayed at pH 4.5 on pulp cells cultured with and without DPH suggests that no significant increase in activity could be found in this experiment. There can be no doubt, though, that enzyme activity assayable at pH 5.2 is selectively induced. This finding explains the original discrepancy between the biochemical and histochemical data.

Drugs such as phenobarbital and diphenylhydantoin (phenytoin) are typically detoxified by mixed function liver microsomal oxidases by first hydroxylation of one phenyl group and then conjugation as a glucuronide [18]. Induction of these enzymes by DPH has been shown by Andreasen and Bremmalgaard [1] who also demonstrated that DPH-treated rats had an increased ethanol-oxidizing activity. Other drugs such as meprobomate may also be metabolized more quickly if DPH is also administered [19]. These findings would suggest that an over-production of these enzymes occurs.

The enzyme responsible for glucuronide conjugation is UDP-glucuronyl transferase [20]. The transferase involved in bilirubin clearance has also been demonstrated to increase with administration of anti-epileptic drugs [21]. βGlucuronidase is responsible for clearing the glucuronide after transport of the drug. In the case of cultured cells, one might expect to find extracellular rather than intracellular β-glucuronidase. However, Hänstrom (personal communication) has shown an increase in intracellular than extracellular β-glucuronidase in gingival explants organ-cultured with DPH. He suggests that this might be a result of decreased permeability of the cell membrane to enzymes.

Increased intracellular β-glucuronidase may have some unexpected ramifications. Its most obvious result would be to produce an excess of glucuronide, and that this occurs *in vivo* is suggested by the increase in glucaric acid excretion in DPH and phenobarbital-treated patients [22, 23]. Glucaric acid is a metabolite of glucuronate; glucuronate is also important to the synthesis of both ascorbic acid and acid mucopolysaccharides.

Glucuronic acid is also an important component of acidic glycosaminoglycans which are associated with connective tissue ground substance. Chondroitin sulfate, hyaluronic acid and dermatan sulfate all have glucuronic acid as a major component. Cheng and Staple [24] have shown that hexosamine content of rat skin is increased after chronic DPH administration.

Patients who have received long-term treatment with DPH may have increased serum levels of alkaline phosphatase [25]. This is particularly true of those who exhibit Dupuytren's disease (thickening and hyalinization of collagen bundles in the hand) [26]. Alkaline phosphatase is often associated with mineralization or cell proliferation.

In dental pulp monolayers, alkaline phosphatase activity usually occurs in areas of greatest cell density. The cultures treated with 27 μg/ml DPH, though, were less dense than the others.

Hassel *et al.* [27] have demonstrated that fibroblasts from gingival biopsies of individuals undergoing long-term diphenylhydantoin therapy (when placed in monolayer culture) produce twice as much collagen as those from control patients. This occurs for several cell generations even thogh no DPH is included in the culture medium. These authors suggest that DPH 'selects for' cells which have these properties and that such a selection process may occur *in vivo*. Induction of microsomal enzymes has been implicated in connective tissue disorders [26] so one may ask whether the ability to respond and detoxify dilantin may not be involved in such a selection process. Perhaps the two features are related.

In conclusion, diphenylhydantoin (0.27, 2.7 or 27 μg/ml) in culture medium of primary bovine dental pulp monolayers induced β-glucuronidase activity which is demonstrable biochemically at pH 5.2. Histochemical techniques demonstrated this increase to be intracellular. Because an overabundance in intracellular β-glucuronidase probably leads to an excess of glucuronide, as suggested by increased glucaric acid excretion in DPH- and phenobarbital-treated patients, an increase in other metabolites of that compound, such as ascorbic acid and acid mucopolysaccharides, may be postulated. High concentrations of DPH in the culture fluid (27 μg/ml) also cause an increase in biochemically demonstrable phosphatase which is not apparent histochemically.

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Influence of colchicine derivatives on lysosomal enzyme release from polymorphonuclear leukocytes and intracellular levels of cAMP after phagocytosis of monosodium urate crystals

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Colchicine and its analogues belong to the group of microtubular inhibitors [1]. Microtubules are involved in the transport towards lysosomes of extracellular material wrapped in vesicles. Drug-induced tubulostasis may lead to reduced digestion of phagocytozed material and could prevent metabolic transformation of this material. These events possibly play a role in the inhibition of the generation of inflammatory mediators [2]. Colchicine does not stabilize isolated lysosomes [3], so its primary action on polymorphonuclear functions (release of lysosomal enzymes and chemotactic factors, motility, etc.) is possibly mediated through its antimicrotubular activity [4].

Some of the colchicine derivatives exhibit similar effects as the parent compound, e.g. demecolcine and deacetylcolchiceine, and are equivalent to colchicine in anti-gout activity [5]. Deacetylcolchiceine is nearly as effective as colchicine in inhibition of urate-stimulated motility of PMN [6] and less active in urate-induced paw swelling in rat [7].

Since enzyme release from PMN is probably crucial for the tissue injury in gouty arthritis [8], the influence of colchicine derivatives—demecolcine and deacetylcolchiceine—on lysosomal enzyme release from PMN after phagocytosis of monosodium urate crystals (MSU) and on the intracellular level of 3′, 5′ cyclic adenosine monophosphate (cAMP).

Demecolcine and deacetylcolchiceine were kindly supplied by Prof. F. Šantavý, M.D., Biochemical Institute of the Medical Faculty, Palacký University, Olomouc, Czechoslovakia.

MSU microcrystals (0.5–30 μm) were a gift from Dr. Továrek, University Hospital, Brno, Czechoslovakia.

Separation of leukocytes [9]. Leukocytes were obtained from the venous blood of healthy young men. Blood (450 ml) was drawn into plastic flasks with 0.9. ml of heparin (5000 U per ml) and 90 ml of a 6% dextran solution. Sedimentation was allowed to proceed in the same flasks for 40 min at room temperature. The cell-rich supernatant was sedimented at 100 *g* for 8 min at room temperature. The erythrocytes were removed by hypotonic lysis (90 ml 0.85% NaCl for 30 sec, 270 ml distilled water added for 20 sec, 90 ml 2.6% NaCl added, and the leukocytes were washed two more times in 0.15 M NaCl and resuspended in the buffered medium to a concentration of 5×10^7 leukocytes per ml medium (1% glucose in phosphate buffered saline, pH 7.4, containing 500 U of heparin per 100 ml.) Neutrophils were 60–75 per cent of total leukocytes.

Measurement of enzyme release. Portions of cell suspension 0.7 ml were dispensed into 10 × 75 mm plastic test tubes. The cells were incubated at 37° with gentle shaking with demecolcine or with deacetylcolchiceine in various concentrations 0.5 ml. Autologous serum was added to a concentration of 10%. After 1 hr incubation the cells were exposed for 1 hr to particles of microcrystalline monosodium urate 0.2 ml. The final concentration of urate was 0.5 mg per ml medium. At the end of experiments, tubes were centrifuged at 755 *g* at 4°. The cell-free supernatant fractions were used for enzyme determination. Portions (0.5 ml) of 0.05 M Tris–HCl buffer (pH 7.5) containing 4 mM EDTA were added to sediments (EDTA acts as a phosphodiesterase inhibitor to prevent degradation of cyclic nucleotides by plasma enzymes). Samples were heated to 100° and centrifuged at low speed. Supernatant fractions were stored at −20° for determination of cAMP.

Estimation of enzymatic activities. Lactate dehydrogenase (EC 1.1.1.27) was determined by the method of Bergmeyer *et al.* [10] and neutral proteases (EC 3.4.4) activity was estimated by the procedure reported by Ignarro [11].

Beta-glucuronidase (EC 3.2.1.31) activity was measured with phenolphthalein glucuronide as substrate [12] and acid phosphatase (EC 3.1.3.2) was established using *p*-nitrophenyl-phosphate as substrate [13].

Determinations of total enzyme activities were made after cells either incubated with tested drugs or without incubation were lysed by six freeze–thaw cycles. Broken cells preparations were centrifuged and enzyme activities were then determined. Incubation with added drugs did not influence the total enzyme activity in comparison with untreated cells.

The cyclic nucleotide levels were estimated by cyclic AMP assay kit (The Radiochemical Centre, Amersham, U.K.) [14, 15].

Polymorphonuclear leukocytes incubated with MSU crystals released lysosomal enzymes as well as cytoplasmic marker enzyme—LDH (Tables 1 and 2). The preincubation with both colchicine derivatives in various concentrations resulted in a decrease of lysosomal enzyme release. The inhibition was dose-dependent. The release of cytoplasmic enzyme LDH tended to decrease but not to such an extent as the release of lysosomal enzymes, especially in the experiment with demecolcine. Phagocytosis of MSU crystals induced the decrease of cAMP level (Table 3). In the presence of tested colchicine derivatives (in concentrations 10^{-3} and 10^{-4} M) there was an elevation in cAMP. This increase was dose-dependent.

The release of lysosomal enzymes may be influenced by several pharmacologic agents [16]. Three major types of compounds have been studied: (1) those that interfere with the levels of cyclic nucleotides—elevation of cAMP cellular levels reduces the enzyme release. Accumulation of cGMP results in enhancement of enzyme release from PMN; (2)