Conversely, the charge neutralization model obviously takes precedence over the substrate depletion model in vesicles in which regulation of the activity of phospholipase A1 by negative charges occurs, as evidenced by the data presented here and the kinetic data reported by Mingeot-Leclercq *et al.* [3]. Further studies, however, will need to establish whether gentamicin binds in a similar fashion and to the same extent to both types of vesicles. Yet, because the composition and probably the organization of biological membranes, especially those of lysosomes, is more akin to that of type 1 than type 2 vesicles, we may already suggest that the model of charge neutralization is more relevant to the *in vivo* situation, and may therefore be of greater help in understanding and, hopefully, avoiding the phospholipidosis induced by aminoglycosides [1]. This model also suggests that the activity of phospholipase A1 may be modulated *in vivo* by variation of the lipid composition and/or the charge of the membranes, a phenomenon that may play an important role in phospholipid turnover in health and disease.

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* Corresponding author: Ms J. Piret, ICP/UCL, Avenue Hippocrate 75, Bte 75.39, B-1200 Brussels, Belgium. Tel. (32)2-764-74-68; FAX (32)2-764-52-94.

*Laboratoire de Chimie Physiologique*
*Groupe de Pharmacologie Cellulaire et Moléculaire*
*Université Catholique de Louvain*
*and International Institute of Cellular and Molecular Pathology*
*Brussels, Belgium*

JOCELYNE PIRET*
BELLAMKONDA K. KISHORE
PAUL M. TULKENS

REFERENCES

1. Laurent G, Kishore BK and Tulkens PM, Aminoglycoside-induced renal phospholipidosis and nephrotoxicity (Commentary). *Biochem Pharmacol* **40**: 2383–2392, 1990.
2. Mingeot-Leclercq MP, Piret J, Brasseur R and Tulkens PM, Effect of acidic phospholipids on the activity of phospholipases and on their inhibition by aminoglycoside antibiotics: I. Biochemical analysis. *Biochem Pharmacol* **40**: 489–497, 1990.
3. Mingeot-Leclercq MP, Laurent G and Tulkens PM, Biochemical mechanism of aminoglycoside-induced inhibition of phosphatidylcholine hydrolysis by lysosomal phospholipases. *Biochem Pharmacol* **37**: 591–599, 1988.
4. Hostetler KY and Jellison EJ, Inhibition of kidney phospholipase A1 by aminoglycosides is a novel variant of substrate depletion inhibition. *J Pharmacol Exp Ther* **254**: 188–191, 1990.

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# Glucocorticoid-mediated potentiation of P450 induction in primary culture of rainbow trout hepatocytes

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**Abstract**—Induction of 7-ethoxyresorufin *O*-deethylase activity (a cytochrome P450IA-dependent activity) by β-naphthoflavone (0.36 μM) is increased 2–3-fold by dexamethasone or cortisol ($10^{-9}$–$10^{-7}$ M) in rainbow trout hepatocyte cultures. This potentiation does not seem to be a time-dependent process and could be a classical glucocorticoid receptor-mediated event resulting in enhanced transcriptional activation of the *CYP*1A, as previously shown in mammals. Since glucocorticoid levels can increase in fish exposed to pollutants, such steroids may interfere with the induction response to xenobiotics.

The hepatic cytochrome P450-dependent monooxygenase system metabolizes a large number of potentially harmful xenobiotics, and is highly inducible by well-known organic pollutants such as polycyclic aromatic hydrocarbons (PAHs*) and polychlorinated biphenyls [1]. This system has been described well in fish species, the cytochrome P450IA (P450IA) isoenzymes being especially sensitive to these pollutants [2, 3]. In mammals, PAH-induced accumulation of P450IA has been shown to involve primarily an increase in transcription mediated by the cytosolic *Ah* receptor but posttranscriptional regulation may also be important [4, 5]. In fish, the factors involved in P450 induction by PAHs are not well characterized, e.g. the presence of an *Ah* receptor was demonstrated only recently [6]. Nevertheless, the induction response has been shown to be modulated by numerous factors such as reproduction stage, sex, age and ambient water temperature. In mammals, GC are important modulators of P450IA induction by PAH-like compounds. Both *in vivo* experiments and studies on cultured hepatocytes show a significant potentiation of the PAH-mediated induction

* Abbreviations: βNF, beta-naphthoflavone; DEX, dexamethasone; DMSO, dimethyl sulfoxide; EROD, 7-ethoxyresorufin *O*-deethylase; GC, glucocorticoids; Hepes, *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid; PAH, polycyclic aromatic hydrocarbon; TAT, tyrosine aminotransferase; P450, cytochrome P450.

by GC [7, 8]. Thus, GC could be a candidate for modulatory effects of P450 induction in fish. Moreover, GC are physiologically important steroids which are elevated in the plasma during stress in fish [9].

The aim of the present study was to investigate the role of GC in regulating P450 induction by β-naphthoflavone (βNF) in rainbow trout liver, using primary cultures of hepatocytes. This system was shown to retain several physiological important properties, including stable and inducible P450 levels during a 5 day culture period, which is important for regulatory studies [10–12].

*Materials and Methods*

*Animals.* Rainbow trout (*Oncorhynchus mykiss*) with an average weight of 250 g, were obtained from a local hatchery near Göteborg. The trout were kept in tanks with aerated, filtered and recirculating freshwater at a temperature of 10° and fed with commercial pellets. All fish were acclimated to laboratory conditions for a minimum of 1 month before experimentation.

*Chemicals.* Medium 199, antibiotic–antimycotic solution (10,000 U penicillin G/mL, 10 mg streptomycin/mL, 25 mg amphotericin B/mL, in 0.9% NaCl), L-glutamine, dexamethasone 21-phosphate and hydrocortisone 21-phosphate (cortisol phosphate), collagenase type IV, were from the Sigma Chemical Co. βNF was purchased from Aldrich and 7-ethoxyresorufin from Boehringer. All other reagents were of analytical grade.

*Isolation of hepatocytes and culture conditions.* Rainbow trout hepatocytes were isolated as described previously [11]. Cell viability was assayed by a dye exclusion test using 0.4% Trypan blue. Each perfusion yielded about 0.5–$1 \times 10^9$ cells, with viability over 90%. All liquids and glassware were sterilized prior to use by filtration or autoclaving. After counting of cells using a Bürker chamber, hepatocytes in culture medium were seeded at a density of $2 \times 10^6$ cells/mL onto surface-treated plastic Petri dishes (Falcon Primaria) and incubated at 12°. In these conditions, hepatocytes attached firmly to culture dishes and formed a rather well-organized monolayer within 24 hr after plating. The hepatocytes were harvested by flushing the Petri dishes with culture medium. Cell suspensions were centrifugated (2 min, 60 *g*), and the pellets were resuspended in 1 mL phosphate buffer (0.1 M), pH 7.4, containing 20% glycerol. Cell suspensions were frozen in liquid nitrogen and stored at −80° prior to assays.

*Experimental treatment.* Glucocorticoids were solved in water and βNF in DMSO (0.1% final, concentration shown to be not cytotoxic) and mixed with the culture medium to the desired concentrations. Controls received DMSO alone. In experiment I, DEX or cortisol were added to the culture medium prior to cell seeding at concentrations ranging from $10^{-9}$ to $10^{-7}$ M. After 24 hr of preculture, βNF (0.36 μM final concentration) and GC were added when the culture medium was changed. NADPH-cytochrome *c* reductase, EROD and TAT activities were measured after 48 hr of exposure. In experiment II, βNF and the glucocorticoids were simultaneously added 1 day after cell seeding and EROD activity measured 48 hr after addition of βNF and GC.

*Enzyme assays.* Cells were lysed by sonication. EROD activity was measured at 20° as described previously [11] using a 7-ethoxyresorufin final concentration of 0.5 μM in phosphate buffer (pH 8.0, 50 mM). TAT activity was measured at 20° following the formation of 4-hydroxybenzaldehyde from L-tyrosine and α-ketoglutarate as described by Granner and Tomkins [13]. NADPH-cytochrome *c* reductase activity was measured as described previously [14].

*Protein assay.* Protein concentration was determined according to Lowry *et al.* [15] using bovine serum albumin as a standard.

*Statistics.* Statistical analyses were performed by the Dunnett's *t*-test [16].

*Results and Discussion*

TAT activity, specifically induced by GC, was increased in cells exposed to $10^{-7}$–$10^{-6}$ M DEX or $10^{-7}$ M cortisol for 48 hr, indicating GC uptake by cultured hepatocytes and subsequent inducive effect of GC (Table 1). However, the lower GC concentrations did not affect TAT activity, but investigations at shorter induction times would be necessary to conclude this, since the response of TAT activity to GC normally occurs earlier than that for the potentiation phenomenon seen during PAH induction of P450IA1, as shown in mammals [7].

The basal EROD activity was not increased by cortisol or DEX (EROD activity mean value: 0.036 nmol/min × mg protein, see Fig. 1), suggesting that GC alone do not have an inducive effect on P450IA. This agrees with results obtained in primary mammalian cell cultures [8, 17].

βNF treatment of hepatocytes for 48 hr resulted in a 5–5.3-fold induction of EROD activity (Fig. 1). Induction ratio of EROD activity in trout hepatocytes is in agreement with other studies [10, 11], but remains low compared to those obtained in cultured rat hepatocytes [1].

When the cells were incubated with DEX ($10^{-9}$–$10^{-7}$ M) for 24 hr before addition of βNF, a potentiation of EROD

Table 1. Induction of TAT by DEX and cortisol

| *In vitro* treatment | TAT activity (nmol *p*-hydroxybenzaldehyde/min × mg protein) |
|---|---|
| None | 0.361 ± 0.083 |
| DEX (M) | |
| $10^{-9}$ | 0.400 ± 0.053 |
| $10^{-8}$ | 0.387 ± 0.012 |
| $10^{-7}$ | 0.481 ± 0.066* |
| Cortisol (M) | |
| $10^{-9}$ | 0.348 ± 0.007 |
| $10^{-8}$ | 0.412 ± 0.087 |
| $10^{-7}$ | 0.612 ± 0.104* |

Cells were incubated with DEX or cortisol for 72 hr after cell seeding and harvested after 48 hr of βNF (0.36 μM) induction. The results from βNF-treated and untreated cells are included since they were not statistically different (each value represents the mean of six dishes of cells ± SEM).

* Significantly different ($P < 0.05$) from the steroid-free control.

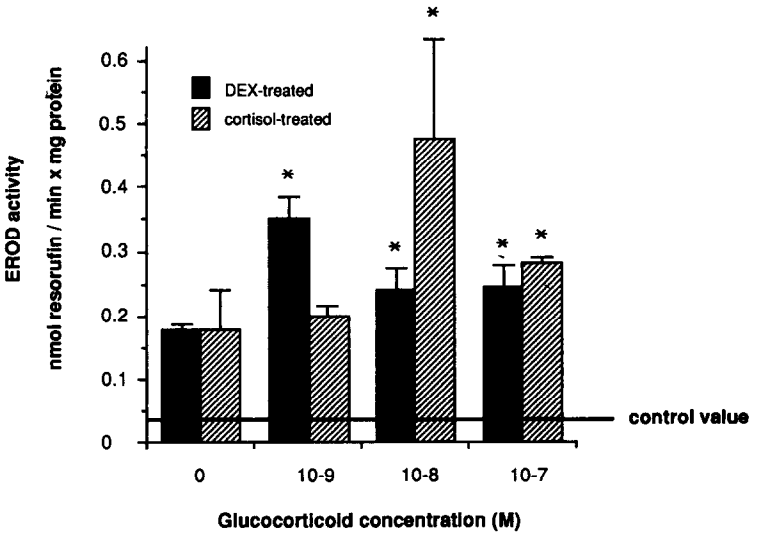


Fig. 1. Potentiation of the βNF induction of EROD activity by DEX and cortisol. DEX or cortisol were added at different concentrations to freshly isolated hepatocytes and βNF 24 hr thereafter. EROD activity was measured after 48 hr of βNF induction (each value represents the mean of three dishes; SEM are shown as vertical bars). Horizontal line gives the mean value of the control (0.036 nmol resorufin/min × mg protein). * Significantly different ($P < 0.05$) from the GC-free assay.

induction was seen resulting in a 6.8–9.8-fold induction of EROD activity (Fig. 1). In the same way, incubation of hepatocytes with cortisol for 24 hr before addition of βNF resulted in a 6–8-fold EROD induction when $10^{-8}$–$10^{-7}$ M steroid was used, whereas no potentiation of EROD induction was seen in cells incubated with $10^{-9}$ M cortisol (Fig. 1). Further investigations have confirmed this potentiation phenomenon in trout hepatocytes when using benzo(*a*)pyrene as an inducer and cortisol (unpublished data). Potentiation of P450IA induction has also been described in rat *in vivo* [7], in fetal rat hepatocyte culture [8, 18, 19] and in cultured human hepatocytes [19, 20]. In experiment II, both DEX and cortisol potentiated the induction of EROD when the steroids and βNF were added at the same time, leading to the same induction increase as in experiment I (data not shown). Potentiation of P450 induction in liver cells of trout may therefore need the steroid and the inducer simultaneously but these results provide little evidence that the GC potentiation of EROD induction in primary cultures of trout hepatocytes requires a time-dependent process as it has been recently demonstrated in Reuber hepatoma cells [21].

To our knowledge, this is the first time that such a GC potentiation of monooxygenase activity induction has been shown in a lower vertebrate. The role of GC in potentiation of PAH-dependent induction of P450IA in rat, human and fish thus may demonstrate a common regulation of this protein induction across vertebrate species. This potentiation could be a classical GC receptor-mediated event resulting in enhanced transcriptional activation of the *CYP*1A, as previously shown in mammals [22, 23]. However, the fact that mechanisms of P450IA potentiation by corticosteroids in mammals have been discussed to involve both transcriptional activation of the P450IA gene as well as stabilization of mRNA or the enzyme [24] has to be taken into account for fish.

NADPH-cytochrome *c* reductase activity was not affected by βNF and/or GC treatment (mean activity value 5.2 nmol/min × mg protein), suggesting that this step is not a rate-limiting step of the observed synergism phenomenon.

The possibility of potentiating P450IA induction in hepatocyte culture by using low cortisol concentrations is relevant to wild fish living in polluted areas. The concentrations of cortisol used in this study are within the physiological range measured in unstressed to stressed trout ($10^{-9}$–$5 \times 10^{-7}$ M) [9]. It is quite important to consider, on an ecotoxicological point of view, this possible regulation by GC of P450 induction. Fish living in polluted areas may respond, at least transiently, with an increase in plasma glucocorticoid levels which may modulate P450 induction. Present work was carried out in fish cell culture and thus involvement of GC in induction regulation remains to be investigated *in vivo*.

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*Laboratoire d'Ecotoxicologie
INRA-ENVL
Ecole Nationale Vétérinaire de
Lyon BP 83
69280 Marcy l'Etoile
France
‡Department of
Zoophysiology
University of Göteborg
PO Box 250 59
S-400 31 Göteborg
Sweden

ALAIN DEVAUX*†
MAIJA PESONEN‡
GILLES MONOD*
TOMMY ANDERSSON‡

† To whom correspondence should be addressed.

REFERENCES

1. Okey AB, Enzyme induction in the cytochrome P-450 system. *Pharmacol Ther* **45**: 241–298, 1990.

2. Kleinow KM, Melancon MJ and Lech JJ, Biotransformation and induction implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. *Environ Health Perspect* **71**: 105–119, 1987.
3. Stegeman JJ, Cytochrome P450 forms in fish: catalytic, immunological and sequence similarities. *Xenobiotica* **19**: 1093–1110, 1989.
4. Silver G and Krauter KS, Expression of cytochromes P-450c and P-450d mRNAs in cultured rat hepatocytes. *J Biol Chem* **263**: 11802–11807, 1988.
5. Pasco DS, Boyum KW, Merchant SN, Chalberg SC and Fagan JB, Transcriptional and post-transcriptional regulation of the genes encoding cytochromes P-450c and P-450d *in vivo* and in primary hepatocyte cultures. *J Biol Chem* **263**: 8671–8676, 1988.
6. Lorenzen A and Okey AB, Detection and characterization of [$^3$H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin binding to Ah receptor in a rainbow trout hepatoma cell line. *Toxicol Appl Pharmacol* **106**: 53–62, 1990.
7. Sherratt GJ, Banet DE, Linder MW and Prough RA, Potentiation of 3-methylcholanthrene induction of rat hepatic cytochrome P450IA1 by dexamethasone *in vivo*. *J Pharmacol Exp Ther* **249**: 667–672, 1989.
8. Sherratt AJ, Banet DE and Prough RA, Glucocorticoid regulation of polycyclic aromatic hydrocarbon induction of cytochrome P450IA1, glutathione *S*-transferases, and NAD(P)H: quinone oxidoreductase in cultured fetal rat hepatocytes. *Mol Pharmacol* **37**: 198–205, 1990.
9. Pickering AD, Pottinger TG and Sumpter JP, Independence of the pituitary-interrenal axis and melanotroph activity in the brown trout, *Salmo trutta* L, under conditions of environmental stress. *Gen Comp Endocrinol* **64**: 206–211, 1986.
10. Vaillant C, Monod G, Valotaire Y and Riviere JL, Measurement and induction of P-450 and monooxygenase activities in a primary culture of rainbow trout (*Salmo gairdneri*) hepatocytes. *CR Acad Sci, Ill* **308**: 83–88, 1989.
11. Pesonen M and Andersson T, Characterization and induction of xenobiotic metabolizing enzyme activities in a primary culture of rainbow trout hepatocytes. *Xenobiotica* **21**: 461–471, 1991.
12. Devaux A, Monod G, Masfaraud JF and Vaillant C, Use of a new coumarin analog in the study of cytochrome P-450 dependent monooxygenase activities in primary culture of rainbow trout hepatocytes (*Oncorhynchus mykiss*). *CR Acad Sci III* **312**: 63–69, 1991.
13. Granner DK and Tomkins GM, Tyrosine aminotransferase (rat liver). *Methods Enzymol* **17**: 633–637, 1970.
14. Monod G, Devaux A and Rivière JL, Characterization of some monooxygenase activities and solubilization of hepatic cytochrome P-450 in two species of freshwater fish, the nase (*Chondrostoma nasus*) and the roach (*Rutilus rutilus*). *Comp Biochem Physiol* **88C**: 83–89, 1987.
15. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Dunnett CW, New tables for multiple comparisons with a control. *Biometrics* **20**: 482–491, 1964.
17. Donato MT, Gomez-Lechon J and Castell JV, Effect of xenobiotics on monooxygenase activities in cultured human hepatocytes. *Biochem Pharmacol* **39**: 1321–1326, 1990.
18. Kremers P, Goujon F, De Graeve J, Van Canfort J and Gielen JE, Multiplicity of cytochrome P-450 in primary fetal hepatocytes in culture. *Eur J Biochem* **116**: 67–72, 1981.
19. Mathis JM, Prough RA, Hines RN, Bresnick E and Simpson ER, Regulation of cytochrome P450c by glucocorticoids and polycyclic aromatic hydrocarbons in cultured fetal rat hepatocytes. *Arch Biochem Biophys* **246**: 439–448, 1986.
20. Mathis JM, Prough RA and Simpson ER, Synergistic induction of monooxygenase activity by glucocorticoids and polycyclic aromatic hydrocarbons in human fetal hepatocytes in primary monolayer culture. *Arch Biochem Biophys* **244**: 650–661, 1986.
21. Wiebel FJ and Cikryt P, Dexamethasone-mediated potentiation of P450IA1 induction in H4IIEC3/T hepatoma cells is dependent on a time-consuming process and associated with induction of the *Ah* receptor. *Chem Biol Interact* **76**: 307–320, 1990.
22. Prough RA, Sherratt AJ, Banet DE, Linder MW, Mathis JM and Simpson ER, Modulation of the polycyclic aromatic hydrocarbon-dependent induction of cytochrome P450IA1 by glucocorticoids. *Drug Metab Rev* **20**: 585–599, 1989.
23. Mathis JM, Houser WH, Bresnick E, Cidlowski JA, Hines RN, Prough RA and Simpson ER, Glucocorticoid regulation of the rat cytochrome P450c (P450IA1) gene: receptor binding within intron I. *Arch Biochem Biophys* **269**: 93–105, 1989.
24. Silver G, Reid LM and Krauter KS, Dexamethasone-mediated regulation of 3-methylcholanthrene-induced cytochrome P-450d mRNA accumulation in primary rat hepatocyte cultures. *J Biol Chem* **265**: 3134–3138, 1990.