Zeitschrift für
Ernährungswissenschaft Z Ernährungswiss 32:176–186 (1993)

Originalbeiträge

Studies on the metabolism of metallothionein and alkaline phosphatase of adult rat primary hepatocyte cultures: role of fetal calf serum and agonists of the phosphoinositide cascade

K. Krämer, A. Markwitan and J. Pallauf

Institut für Tierernährung, Justus-Liebig-Universität Gießen

Untersuchungen zum Metabolismus von Metallothionein und alkalischer Phosphatase adulter primärer Rattenhepatocyten: Rolle von foetalem Kälberserum und Agonisten des Phosphoinositol-Effektorsystems

Summary: Adult rat primary hepatocytes maintained in DMEM/F12 (Ham) media were used as a model system for studying the role of fetal calf serum (FCS) and agonists of the phosphoinositide cascade in the metabolism of metallothionein (MT) and alkaline phosphatase (ALP). Experiments were performed both after a 24 h preincubation with FCS and with bovine serum albumin (BSA). Hepatocytes were treated with dexamethasone (DEX), zinc (Zn) and with the agonists of the phosphoinositide cascade A 23187, 1,2-dioctanoyl-sn-glycerol (DiC_8), 12-O-tetradecanoylphorbol-13-acetate (TPA), angiotensin II (AT), platelet activating factor (PAF), Arg^8-vasopressin (VP) and were analyzed for MT and ALP activity in cell homogenates. Cell viability was evaluated by lactate dehydrogenase (LDH) liberation into culture medium, induction of tyrosine aminotransferase (TAT) through DEX and by trypan blue exclusion. Overall, cell viability was improved by the FCS pretreatment and by DEX. Exposure of hepatocytes to the established direct inducers Zn and DEX of MT resulted in a manifold increase in MT, independent of whether the cultures were FCS pretreated or not. The FCS preincubation produced a moderate elevation of ALP activity by stimulating cell viability. However, ALP was unaltered in response to Zn and DEX. None of the experiments conducted with agonists of the phosphoinositide cascade led to an elevation of MT and ALP. Only the incubation of hepatocytes with A 23187 resulted in a concentration dependent significant decrease of MT and ALP. This observation was due to a cytotoxic effect of A 23187, displayed by LDH leakage and an increase in the number of cells stained with trypan blue.

Abbreviation index:

ALP	alkaline phosphatase	MT	metallothionein
AT	angiotensin II	ns	not significant
A 23187	calciumionophore	P	probability (level of significance)
BSA	bovine serum albumin	PAF	platelet activating factor
DEX	dexamethasone	PBS	phosphate buffered saline
DiC_8	1,2-dioctanoyl-sn-glycerol	r	coefficient of correlation
DMSO	dimethyl sulfoxide	TAT	tyrosine aminotransferase
FCS	fetal calf serum	TPA	12-O-tetradecanoylphorbol-13-acetate
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid	TRIS	tris(hydroxymethyl)aminomethane
IU	international unit	U	unit
LDH	lactate dehydrogenase	VP	Arg^8-vasopressin

In conclusion, in primary hepatocyte cultures agonists of the phosphoinositide did not have an effect on the metabolism of MT and ALP. Previous in vivo results indicating alterations of Zn metabolism in liver, therefore seem to be caused by indirect systemic responses.

Zusammenfassung: Am Modell primärer Rattenhepatocytenkulturen wurde die Beteiligung von Agonisten des Phosphoinositol-Effektorsystems am Metabolismus von Metallothionein (MT) und alkalischer Phosphatase (ALP) untersucht. Alle Experimente wurden in DMEM/F12 (Ham)-Medium sowohl nach 24stündiger Vorinkubation mit foetalem Kälberserum (FCS) als auch nach Vorinkubation mit Rinderserumalbumin (BSA) durchgeführt. Die Versuche an den Hepatocytenkulturen wurden mit Dexamethason (DEX), Zink (Zn) und den Agonisten des Phosphoinositol-Effektorsystems, der Calciumionophore A 23187, 1,2-Dioctanoyl-sn-glycerol (DiC_8), 12-O-Tetradecanoylphorbol-13-acetat (TPA), Angiotensin II (AT), Platelet Activating Factor (PAF) und Arg^8-Vasopressin (VP), durchgeführt. Als Parameter wurden die Konzentrationen an MT und die Aktivität der ALP im Zellmaterial bestimmt. Die Vitalität der Kulturen wurde über die Freisetzung der Aktivität der Laktatdehydrogenase (LDH) ins Kulturmedium, der Induzierbarkeit der Tyrosinaminotransferase (TAT) durch DEX und der Anfärbbarkeit der Zellen mit Trypanblau nachgewiesen. Die Zellvitalität wurde durch die FCS-Vorinkubation und DEX-Supplementierung insgesamt verbessert. Unabhängig davon, ob die Zellen mit FCS oder BSA vorinkubiert wurden, stieg der MT-Gehalt der Zellen durch Zn und DEX, als aus der Literatur bekannte direkte Induktoren von MT, um ein Mehrfaches an. Nach FCS-Vorbehandlung war ein moderater Anstieg der ALP-Aktivität nachzuweisen, der jedoch als Vitalitätseffekt interpretiert werden kann. DEX und Zn führten zu keinen Veränderungen der ALP-Aktivität. Alle getesteten Agonisten des Phosphoinositol-Effektorsystems konnten weder MT noch ALP induzieren. Lediglich A 23187 führte zu einer signifikanten konzentrationsabhängigen Reduktion der beiden Parameter. Diese Beobachtung wurde, durch den Anstieg der LDH-Aktivität im Medium und der Zunahme mit Trypanblau anfärbbaren Zellen, auf einen cytotoxischen Effekt von A 23187 zurückgeführt.

Die vorliegende Untersuchung zeigt, daß Agonisten des Phosphoinositol-Effektorsystems nicht in der Lage sind, den Metabolismus von MT und ALP primärer Rattenhepatocyten zu verändern. Die Ergebnisse früherer in vivo Experimente, in denen Agonisten des Phosphoinositol-Effektorsystems den Zn-Stoffwechsel der Leber modulierten, können somit als indirekter systemischer Effekt gedeutet werden.

Key words: Rats – hepatocytes – phosphoinositide cascade – zinc – metallothionein – alkaline phosphatase

Schlüsselwörter: Ratten – Hepatocyten – Phosphoinositol-Effektorsystem – Zink – Metallothionein – alkalische Phosphatase

Introduction

Mammalian metallothioneins (MT) are a group of polypeptides, containing 61 to 62 amino acid residues, among them 20 cysteines bind seven equivalents of bivalent metal ions in two clusters. It has been suggested that MT is involved in Zn homeostasis, in Cd detoxification, in supplying metalloenzymes with Zn, and also functions as a radical scavenger. Hepatic MT is induced in vivo by metals (Zn, Cu, Cd), glucocorticoids, catecholamines, polypeptide hormones and in response to acute-phases such as chemicals, cold, infections and starvation. For reviews see Cousins (7, 8) and Bremner and Beattie (6). The mechanism of MT induction can either be a direct activation of the genome (metals and glucocorticoids) or an indirect response to acute-phases. Previous whole animal experiments (4, 5, 16, 25) showed that rat hepatic MT is induced by agonists of the phosphoinositide cascade. In one of these studies (25) a marked decline in feed intake, which is also known to lead to MT induction (18, 26, 33), was observed in response to *ip*-application of synthetic agonists of the phosphoinositide cascade. These findings demonstrate that MT synthesis is susceptible to complex systemic effects and that in vivo approaches can not elaborate whether a hormone or agonist is directly involved in the

regulation of MT. Primary cultures of adult rat hepatocytes are a useful model for studying mechanistic aspects of MT synthesis. In contrast to immortalized cell lines, primary hepatocytes are differentiated and have conserved many of the in vivo metabolic and structural features (2, 38).

In the present investigation, hepatocyte monolayer cultures were utilized as a model to examine at the cellular level the role of agonists of the phosphoinositide cascade in the metabolism of MT and ALP. This in vitro system was adopted because whole animal experiments failed to distinguish between a direct agonist effect and fasting conditions. In addition to MT synthesis the activity of the zinc-metallo-enzyme alkaline phosphatase (EC 3.1.3.1, ALP) was evaluated in hepatocytes. It is well known that ALP under experimentally defined conditions in animal experiments reacts as a sensitive marker of Zn status (24).

Materials and Methods

Isolation and cultivation of hepatocytes

All chemicals exposed to the hepatocytes were, if available, cell culture tested and sterile or at least reagent grade. Sterilization of nonsterile chemicals and materials was performed by dry and wet heat (autoclaving) or for heat sensible collagenase enzyme and hormones through sterile filtration. Hepatocytes were isolated by a modification of the two-step collagenase perfusion procedure described by Seglen (35). All buffers used were equilibrated with a gas mixture composed of 5 % CO_2 and 95 % O_2.

Liver parenchymal cells were isolated from 3–4 month-old rats (250–300 g) from the Institute's breeding colony. The animals were housed under environmentally controlled conditions (12 h light-dark cycle, 22 °C, 55 % humidity). Rats were given free access to a commercial rat chow (ALTROMIN, Lage) and tap water prior to experimentation. Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (90 mg/kg body weight). The abdomen was opened and 0.5 mL (2500 IU) sodium heparin (ROCHE, Grenzach-Wyhlen) were dropped onto the mesentery to prevent blood clotting. The intestine was displaced to inspect the portal vein and inferior *vena cava*. Loose ligatures were tied around the portal vein and *vena cava inferior*. An incision was made along the *vena porta* and a catheter introduced into the vein which was connected via a peristaltic pump to a reservoir with perfusion buffer 1 [107.9 mM NaCl, 6.7 mM KCl, 8.5 mM NaOH, 25 mM HEPES, 5 mM glucose, 25 mM $NaHCO_3$, 0.01 % phenol red, pH 7.4] at 37 °C. The *vena cava inferior* was severed for perfusate outflow and the catheter fixed by tightening the ligature. While the thoracic cavity was opened the liver was perfused *in situ* at a flow rate of 20 mL/min. The thoracic *vena cava* was cut and the flow rate increased to 30 mL/min. A second catheter was inserted into the thoracic *vena cava* and secured by a ligature. After 200 mL of perfusion buffer 1 had passed the liver it was changed to perfusion buffer 2 [perfusion buffer 1 plus 3 mM $CaCl_2$ and 0.1 % collagenase H (Boehringer–Mannheim)]. The intercellular junctions were dissolved by recirculating 100 mL of this enzyme-containing buffer through the liver for 12 min at 30 mL/min. The liver was cut off with care and the liver cells were liberated into the ice-cold perfusion buffer 1 by gently raking with scissors.

Parenchymal cells were separated from nonparenchymal cells and vascular debris in three washing steps. The initial cell suspension was filtered through a nylon gauze (150 μm pore size), centrifuged (63 g, 2 min), the supernatant discarded, the pellet suspended in buffer 1, filtered through a 80 μm nylon mesh and pelleted again. The washing was repeated without filtration in ice-cold DMEM/F12 (Ham) medium with glutamine

and HEPES (GIBCO, Eggenstein) and resuspended in DMEM/F12 medium supplemented with 10^{-7} M insulin, 5 mg/L transferrin and 50 mg/L gentamycin (Boehringer – Mannheim), subsequently termed as basal medium. Cells were counted and cell viability was estimated by trypan blue exclusion in a hemocytometer (Fuchs–Rosental). Routinely, $> 100 \times 10^6$ cells/100 g body weight were yielded and the viability was assessed > 75 %. Cells were finally diluted in basal medium containing 10 % (v/v) fetal calf serum (FCS) (Boehringer – Mannheim) to obtain 1.1×10^6 cells/mL. Collagen-coated, 60-mm, polystyrene culture dishes were inoculated with 2 mL aliquots of the final cell suspension. Collagen coating of culture dishes was performed according to (37) with rat-tail collagen (SERVA, Heidelberg). Culture dishes were incubated at 37 °C (98 % air/2 % CO_2, 95 % humidity). After a 70-min incubation period, the medium with unattached cells was aspirated and the cultures were washed with 3 mL of phosphate buffered saline (PBS) [136.9 mM NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4 \times 2\ H_2O$, 1.5 mM KH_2PO_4, pH 7.4]. Depending on experimental design, cells were cultured in 3 mL fresh basal medium, either supplemented with 10 % (v/v) FCS (Myclone plus, GIBCO, Eggenstein) or 2 g/L bovine serum albumin (BSA) (Boehringer – Mannheim) for further 24 h. In this 24-h equilibration period the hepatocytes adapted to in vitro conditions, recovered from the isolation stress, and flattened to a monolayer.

Experimental design

After 24 h the medium was changed and experiments initiated by adding agonists of the phosphoinositide cascade (0.5 % of volume). The Zn concentrations of the media were approximately 1–2 μM for the BSA and 5 μM for the FCS medium. Control dishes were supplemented with the solvents. Experiments were performed both with FCS and BSA incorporation into the medium during the 24 h equilibration period. All incubations usually lasting 24 h were done in triplicate from two independent cell preparations. Agonists and their concentrations tested are listed below:

Dexamethasone (DEX) (SIGMA, Deisenhofen) in 5 % (v/v) DMSO/PBS	1 μM
$ZnSO_4 \times 7\ H_2O$ (SIGMA, Deisenhofen) in H_2O	80 μM
Calciumionophore (A 23187) (SIGMA, Deisenhofen) in DMSO	0.1, 1, 5 μM
1,2-Dioctanoyl-sn-glycerol (DiC_8) (SIGMA, Deisenhofen) in DMSO	0.1, 1, 10 μM
12-O-Tetradecanoylphorbol-13-acetate (TPA) (SERVA, Heidelberg) in DMSO	0.1, 1, 10 μM
Angiotensin II (AT) (BACHEM, Heidelberg) in PBS	0.1, 1, 10 μM
Platelet activating factor (PAF) (BACHEM, Heidelberg) in 5 % (v/v) DMSO/PBS	0.1, 1, 10 μM
(Arg^8)-Vasopressin (VP) (BACHEM, Heidelberg) in PBS	0.1, 1, 10 μM

Sample preparation and analytical methods

At the end of the experiments, the supernatant media were transferred into plastic tubes. The monolayers were rinsed with 3 mL PBS and harvested into 2 mL of TRIS-HCl buffer [10 mM, pH 7.4] with a rubber policeman. All samples were stored until analysis at –25 °C. Homogenization was performed after the addition of a protease inhibitor mixture [in final concentrations 0.3 mM phenylmethylsulfonyl fluoride (SERVA, Heidelberg) and 1 mM benzamidine hydrochloride (SIGMA, Deisenhofen)] by sonication (Labsonic U, BRAUN, Melsungen) for 1 min at 60 W and 0.5 s duty cycle.

Metallothionein in hepatocyte homogenates was quantified by the ^{109}Cd-hemoglobin-binding assay (13) using a high specific activity Cd solution [1.15 μCi ^{109}Cd (carrier free, Amersham, Braunschweig) and 1.5 μg Cd/mL in 10 mM TRIS-HCl, pH 7.4] and 20 000 g for centrifugation of samples. Activity of ALP in cell homogenates was estimated with p-nitrophenylphosphate as a substrate (12). Cell viability was assessed by the activity of

the cytosolic enzyme lactate dehydrogenase (EC 1.1.1.27, LDH) liberated into the culture medium (11). In addition to membrane leakage of LDH, a further criterion for cell viability, the induction of tyrosine aminotransferase (EC 2.6.1.5, TAT) by 1 μM DEX was determined (15). Protein concentration was evaluated according to the Lowry-Folin method (10). Media zinc was analyzed by atomic absorption spectrophotometry in an air-acetylene-flame (Philips PU 945, Kassel).

Statistical analysis

In the experiments with DEX and Zn, data obtained either with or without FCS pretreatment were compared by analysis of variance. When the analysis indicated a significant difference, treatment effects were determined by comparison to appropriate controls by Bonferroni's test for multiple comparisons. Results from agonists of the phosphoinositide cascade were processed by a linear regression model ($Y = aX + b$), evaluating a dependence on agonist concentration. Differences were considered significant if $p < 0.05$.

Results and Discussion

Fetal calf serum stabilizes and stimulates the growth of cell cultures through intrinsic hormones, attachment factors and growth factors. It can also function as a pH buffer and by inactivating liberated proteases from damaged cells (1, 29). But these characteristics were also open to criticism because FCS-containing medium lacks defined culturing conditions (1). Under defined conditions in serum-free media FCS is replaced by insulin as a growth-stimulating hormone and by transferrin and BSA to maintain essential transport functions (1, 30). The experiments in the present investigation were carried out both with (FCS) and without FCS (BSA) preincubation, (1) to operate FCS-free during the exposition of the agonist, avoiding a masking effect of serum on agonists and (2) to take advantage of a long-term cell culture stabilizing effect of FCS, which can also modulate the effect of supplemented hormones (36).

Fig. 1 presents the MT content (A) and activity of ALP (B) of hepatocyte cultures after a 24-h treatment with 80 μM Zn and 1 μM DEX. The experiments were both performed after a 24-h preincubation period with BSA and FCS. Both pretreatments resulted in a significant increase of MT in response to the primary inducers Zn and DEX. However, the effect of the supplements was more pronounced under serum-free conditions. Similar results in hepatocyte cultures were previously reported by others (3, 9). The FCS pretreatment produced a moderate increase in cellular ALP activity. This observation can be attributed to a more vital monolayer in response to FCS documented by a decrease in LDH activity of media (Fig. 1C). Cell viability stimulated by FCS supports cell-cell contact of hepatocytes and the reconstruction of bile canaliculi, where liver ALP is mainly localized (23, 40). This also explains the higher specific ALP activity in hepatocyte culture as compared to fresh liver. In contrast to the zinc-metallo-enzyme δ-aminolevulinic acid dehydratase (34), ALP activity of cultured hepatocytes did not react to the media zinc content (Fig. 1B). Differing from results obtained with cultured cancer cells, where ALP increased manifold in response to glucocorticoids (17), in the present investigation ALP activity remained unaltered under the influence of DEX.

Fig. 1C shows the LDH activity in culture medium as a parameter of membrane integrity for the Zn and DEX treatments. The FCS pretreatment produced a 10-fold decrease of control LDH activities. The increase of medium zinc concentration from basal to 80 μM had no effect on membrane leakage after BSA pretreatment, but reduced it

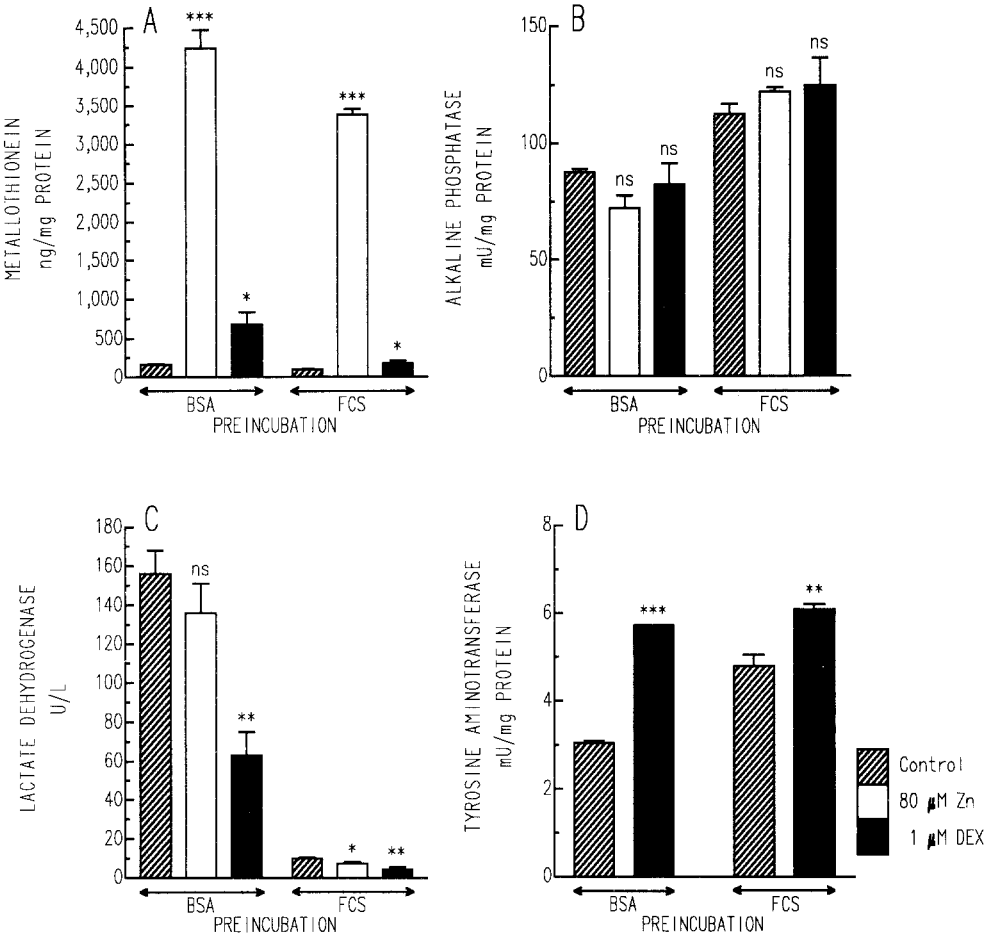

Fig. 1. Metallothionein content (A), activity of alkaline phosphatase (B) and tyrosine aminotransferase (D) in rat hepatocytes and activity of lactate dehydrogenase (C) in culture media following a 24-h treatment with zinc (Zn) and dexamethasone (DEX). Hepatocytes were cultured in DMEM/F12 (Ham) medium and preincubated for 24 h either with bovine serum albumin (BSA) or fetal calf serum (FCS). Results are presented as mean ± standard deviation. Asterisks indicate a significant difference of treatment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) from the representative control by Bonferroni's test

moderately after FCS preincubation. This result confirms a previous observation, that supraphysiological Zn concentrations had no detrimental effects on hepatocyte viability (34). A significant decline of LDH liberation into the medium was observed in response to the supplementation of 1 µM DEX, independent of whether the cultures were pretreated with BSA or FCS, and sustains the hepatocyte stabilizing effect of DEX described earlier (27).

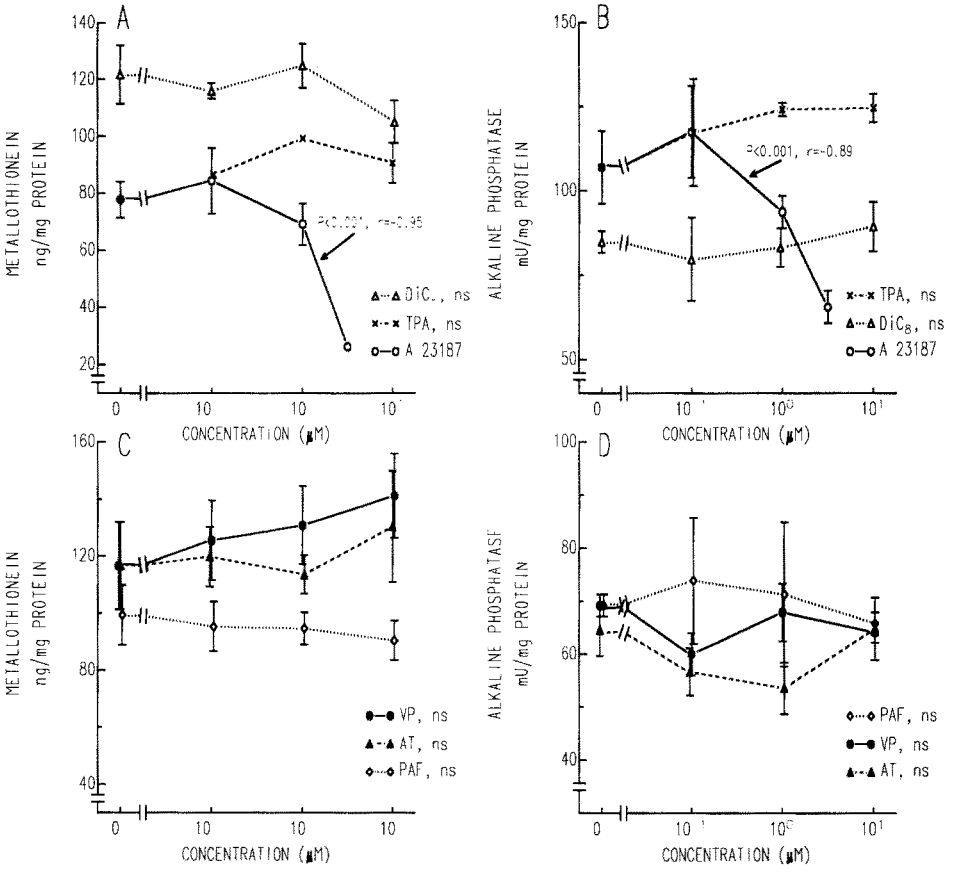

Fig. 2. Metallothionein content (A and C) and activity of alkaline phosphatase (B and D) in rat hepatocytes following a 24-h treatment with A 23187, 1,2-dioctanoyl-sn-glycerol (DiC_8), 12-O-tetradecanoylphorbol-13-acetate (TPA), angiotensin II (AT), platelet activating factor (PAF) and Arg^8-vasopressin (VP). Hepatocytes were cultured in DMEM/F12 (Ham) medium and preincubated for 24 h with bovine serum albumin (BSA). Points represent mean ± standard deviation. Significant effects of agonist concentration on parameters are indicated by the significance level P and coefficient of correlation r, estimated by linear regression

A well documented parameter of liver function, the induction of TAT activity by DEX (20, 38), is shown in Fig. 1D. Similar to the elevation of MT (Fig. 1A) the degree of TAT induction was more marked (2-fold vs. 1.3-fold) in the case of serum-free preincubation. It appears that the FCS pretreatment, although it has a beneficial effect on cell viability, impairs the response to an inducer. In the case of serum pretreatment of TAT samples, it

can also be argued that TAT is induced by intrinsic factors of FCS that mask the DEX effect.

The influence of a 24-h treatment of synthetic and natural agonists of the phosphoinositide cascade on MT content and activity of ALP of hepatocyte cultures is shown in Figs. 2A–2D. All results presented herein were obtained after a serum-free pretreatment of the cells. The effects of agonists on MT and ALP after the FCS preincubation (not shown) were fairly identical. In the phosphoinositide cascade the synthetic agonists DiC_8 and TPA function as activators of proteinkinase C, the calciumionophore A 23187 by increasing cytosolic free Ca^{2+}-concentration. AT, PAF and VP as natural hormones of the phosphoinositide cascade act via a phospholipase C by activation of both pathways of this signal transduction mechanism. (For details see ref. (31).) In all experiments no increase of either MT or ALP by the agonists tested could be detected. Only A 23187 resulted in a concentration-dependent significant decrease in MT content and ALP activity of the hepatocytes (Figs. 2A and 2B). Thus, A 23187 also increased the number of cells stained with trypan blue and elevated LDH activity of media up to 10-fold (control 95 U/L, 5 μM A 23187 1026 U/L), the decline in MT and AP can be interpreted as a toxic effect of this chemical. The FCS preincubation mitigated the harmful effect of A 23187 on cell viability. In contrast to the present results, 7 μM A 23187 caused a moderate increase in MT mRNA in HepG2 cells (human hepatocyte carcinoma) and no detrimental effects on cell viability were reported by the authors (21). In the present study medium LDH activity and viability were unaltered in response to all other agonists examined. The LDH-activities in experiments with BSA-preincubation averaged (highest agonist concentration) 88 U/L for DiC_8 (control 95 U/L), 89 U/L for TPA (control 76 U/L), 131 U/L for AT (control 156 U/L), 98 U/L for PAF and 105 U/L for VP with 113 U/L in the control group. However, the inefficacy of agonists to alter MT and ALP cannot be due to a defect in the expression of membrane receptors. This was proved in a separate investigation with similar concentrations of the agonists tested in the present study through hormone-stimulated elevation of cytosolic free Ca^{2+}-concentration, determined by Fura 2AM fluorescence quench (28). The concentrations of agonists tested in the present investigation also increased cytosolic free Ca^{2+}-concentration in experiments with hepatocytes in suspension culture (14).

To our knowledge, up to now all experiments on the influence of agonists of the phosphoinositide cascade on MT and ALP have been performed with whole animals and with cell lines. Previous in vivo studies with rats demonstrated a manifold induction of hepatic MT in response to AT and norepinephrine as well as TPA and A 23187 (4, 5, 16, 25). However, serum ALP activity was significantly reduced in response to TPA and A 23187 (25), but this alteration was later attributed to a simultaneously observed anorexia reducing serum ALP of intestinal origin (26).

In previous studies with liver cell lines of human and rat origin as well as HeLa cells (human cervix carcinoma) MT-mRNA content was increased by TPA, DiC_8 and A 23187 (21, 22). Palmiter (32) pointed out that translational regulation of MT synthesis plays a minor role, suggesting that MT protein synthesis is proportional to MT-mRNA formation. Thus, the discrepancies between our own experiments and those obtained with cell lines show evidence that MT gene expression is differently controlled in primary hepatocyte cultures and cell lines.

Few data exist on the modification of ALP activity in cell culture by agonists of the phosphoinositide cascade. Only TPA has been examined and it had no effect in a normal rat liver epithelial and in a neoplastic transformed human endometrial cell line (19, 39).

The results in literature and those in the present study indicate that agonists of the phosphoinositide cascade are not involved in the regulation of ALP activity.

When the findings in this investigation and those in literature are taken together, it can be concluded that the culturing conditions were suitable for studying hormonal effects, proved by established responses of MT and TAT to DEX. Agonists of the phosphoinositide cascade did not modulate metabolism of MT and ALP of hepatocytes. Earlier in vivo results indicating an increase of liver MT and a decrease of serum ALP activity caused by agonists of the phosphoinositide cascade can now be explained by an indirect physiological response similar to the MT synthesis in acute-phases. In general, primary hepatocyte cultures are a suitable tool for studying mechanistic aspects of liver function, particularly if complex systemic metabolic regulation can be expected.

Acknowledgements

This study was supported by a research grant from the Deutsche Forschungsgemeinschaft. We thank Mrs. Ute Blindenbacher for her skillful technical assistance and the central department of the Strahlenzentrum of the University of Giessen for giving us the opportunity to work in their laboratories.

References

1. Barnes D, Sato G (1980) Methods for growth of cultured cells in serum-free medium. Anal Biochem 102:255–270
2. Bissel DM, Guzelian PS (1980) Phenotypic stability of adult rat hepatocytes in primary monolayer culture. Ann NY Acad Sci 349:85–98
3. Bracken WM, Klaassen CD (1987) Induction of metallothionein in rat primary hepatocyte cultures: evidence for direct and indirect induction. J Tox Environ Health 22:163–174
4. Brady FO, Helvig BS (1984) Effect of epinephrine and norepinephrine on zinc thionein levels and induction in rat liver. Am J Physiol 247:E318–E322
5. Brady FO, Helvig BS, Funk AE, Garrett SH (1987) The involvement of catecholamines and polypeptide hormones in the multihormonal modulation of rat hepatic thionein levels. In: Kägi JHR, Kojima Y (eds) Metallothionein II. Experientia Supplementum, Vol 52. Birkhäuser, Basel, pp 555–563
6. Bremner I, Beattie JH (1990) Metallothionein and trace minerals. Ann Rev Nutr 10:63–83
7. Cousins RJ (1985) Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiol Rev 65:238–309
8. Cousins RJ (1989) Systemic transport of zinc. In: Mills CF (ed) Zinc in Human Biology. Springer, London, pp 79–93
9. Cousins RJ, Coppen DE (1987) Regulation of liver zinc metabolism and metallothionein by cAMP, glucagon glucocorticoids and suppression of free radicals by zinc. In: Kägi JHR, Kojima Y (eds) Metallothionein II. Experientia Supplementum, Vol 52. Birkhäuser, Basel, pp 545–553
10. Dawson RMC, Elliott DC, Elliott WH, Jones KM (1986) Data for Biochemical Research. 3d ed, Clarrendon Press, Oxford, p 543
11. Deutsche Gesellschaft für Klinische Chemie (1970) Standardisierung der Methoden zur Bestimmung von Enzymaktivitäten in biologischen Flüssigkeiten. Z Klin Chem Klin Biochem 8:188–198, 658–660
12. Deutsche Gesellschaft für Klinische Chemie (1972) Standard-Methode zur Bestimmung der alkalischen Phosphatase (AP). Z Klin Chem Klin Biochem 10:191
13. Eaton DL, Toal BF (1982) Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. Tox Appl Pharm 66:134–142
14. Exton JH (1988) The roles of calcium and phosphoinositides in the mechanism of α_1-adrenergic and other agonists. Rev Physiol Biochem Pharmacol 111:117–224
15. Granner DK, Tomkins GM (1970) Tyrosine aminotransferase (rat liver). In: Colowick SP, Kaplan N (eds) Methods in Enzymology, Vol XVIIA, Academic Press, New York, pp 633–637

16. Helvig BS, Brady FO (1984) Effects of glucagon, Arg-vasopressin, and angiotensin II on rat hepatic zinc thionein levels. Life Sci 35:2513–2518
17. Herz F (1984) Induction of alkaline phosphatase expression. In: Stigbrand T, Fishman WH (eds) Human alkaline phosphatases. Alan Liss, New York, pp 139–166
18. Hidalgo J, Borrás M, Garvey JS, Armario A (1990) Liver, brain, and heart metallothionein induction by stress. J Neurochem 55:651–654
19. Holinka CF, Hata H, Kuramoto H, Guripe E (1986) Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa line). Cancer Res 46:2771–2774
20. Horiuti Y, Nakamura Y, Ichihara A (1982) Role of serum in maintenance of functional hepatocytes in primary culture. J Biochem 92:1995–2000
21. Imbra RJ, Karin M (1987) Metallothionein gene expression is regulated by serum factors and by activators of protein kinase C. Mol Cell Biol 7:1358–1363
22. Karasawa M, Hosoi J, Hashiba H, Nose K, Tohyama C, Abe E, Suda T, Kuroki T (1987) Regulation of metallothionein gene expression by 1α,25-dihydroxyvitamin D_3 in cultured cells and in mice. Proc Natl Acad Sci USA 84:8810–8813
23. Kato S, Aoyama K, Nakamura T, Ichihara A (1979) Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. III. Changes of enzyme activities on cell membrane during culture. J Biochem 86:1419–1425
24. Kirchgessner M, Roth H-P (1983) Nutritional influence of zinc on the activity of enzymes and hormones. In: Sigl H (ed) Metal Ions in Biological Systems. Vol 15, Zinc and its Role in Biology and Nutrition. Marcel Dekker, New York, pp 363–414
25. Krämer K, Markwitan A, Montigel C, Pallauf J (1992) Einfluß eines Aktivators der Proteinkinase C (TPA) und eines Ca^{2+}-mobilisierenden Agonisten (A 23187) auf den Zinkmetabolismus der Ratte. Z Ernährungswiss 31:246–257
26. Krämer K, Markwitan A, Menne A, Pallauf J (1993) Zinc metabolism in fasted rats. J Trace Elem Electrolytes Health Dis (in press)
27. Laishes BA, Williams GM (1976) Conditions affecting primary cell cultures of functional adult rat hepatocytes. II. Dexamethasone enhanced longevity and maintenance of morphology. In Vitro 12:821–832
28. Lehnert V, Weiss C, Weintraut H, Pallauf J (1993) Bestimmung des freien cytosolischen Ca^{2+}-Spiegels in isolierten Rattenhepatocyten mit Hilfe der digitalen Bildanalyse unter Verwendung des Fluoreszenzmarkers Fura 2. Proc Soc Nutr Physiol 1:107
29. Lindl T, Bauer J (1989) Zell- und Gewebekultur. 2. Aufl., Gustav Fischer, Stuttgart
30. Maurer HR (1986) Towards chemically-defined, serum-free media for mammalian cell culture. In: Freshney RF (ed) Animal Cell Culture. A Practical Approach. IRL Press, Oxford, pp 13–30
31. Michell RH, Drummond AH, Downes CP (eds) (1989) Inositol Lipids in Cell Signalling. Academic Press, London
32. Palmiter RD (1987) Molecular biology of metallothionein gene expression. In: Kägi JHR, Kojima Y (eds) Metallothionein II. Experientia Supplementum, Vol 52. Birkhäuser, Basel, pp 63–80
33. Sato M, Mehra RK, Bremner I (1984) Measurement of plasma metallothionein-I in the assessment of zinc status of zinc-deficient and stressed rats. J Nutr 114:1683–1689
34. Schroeder JJ, Cousins RJ (1991) Maintenance of zinc-dependent hepatic functions in rat hepatocytes cultured without added zinc. J Nutr 121:844–853
35. Seglen PO (1976) Preparation of isolated rat liver cells. Meth Cell Biol 13:29–83
36. Sorimachi K, Hayashi T, Takaoka T, Niwa A, Yasumura Y (1988) Requirement of serum pretreatment for induction of alkaline phosphatase activity with prednisolone, butyrate, dibutyryl cyclic adenosine monophosphate and NaCl in human liver cells continuously cultured in serum-free medium. Cell Struc Funct 13:1–11
37. Strom SC, Michalopoulos G (1982) Collagen as a substrate for cell growth and differentiation. Methods Enzymol 82:544–555
38. Tanaka K, Sato M, Tomita Y, Ichihara A (1978) Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. I. Hormonal effects on cell viability and protein synthesis. J Biochem 84:937–946

39. Tsao M-S, Nelson KG, Grisham JW (1984) Biochemical effects of 12-O-tetradecanoylphorbol-13-acetate, retinoic acid, phenobarbital, and 5-azacytidine on a normal rat liver epithelial cell line. J Cell Physiol 121:1–6
40. Watanabe J, Kanamura S, Asada-Kubota M, Kanai K, Oka M (1983) Observation of cytochemical alkaline phosphatase on the plasma membrane of cultured rat hepatocytes by backscattered electron imaging. Anat Rec 207:635–642

received December 10, 1992
accepted February 27, 1993

Authors' address:

Prof. Dr. J. Pallauf, Justus-Liebig-Universität, Institut für Tierernährung, Senckenbergstr. 5, 35390 Gießen, FRG