Z Ernährungswiss 34: 293–300 (1995)

ORIGINAL CONTRIBUTION

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Influence of cAMP-effector-agonists on the synthesis of metallothionein in rat primary hepatocytes

Untersuchungen zum Einfluß von cAMP-Effektoragonisten auf die Synthese von Metallothionein in primär kultivierten Rattenhepatocyten

Summary The model of rat primary hepatocytes incubated in DMEM/F12 (Ham) medium was used for studying the influence of the cAMP-effectors epinephrine (100 µM), norepinephrine (100 µM), glucagon (1 µM) and isoproterenol (1–1000 µM) as well as the synthetic cAMP-analogon dibutyryl-cAMP on the metabolism of metallothionein. Liver parenchymal cells isolated by a two-step collagenase perfusion were incubated with DMEM/F12 containing 5 % (v/v) fetal calf serum (FCS) and 20 µM zinc in Petri dishes. Experiments were initiated after a 24 h equilibration period by adding the agonists for 18 h. MT in hepatocyte homogenates was quantified by the ^{109}Cd-hemoglobin-binding assay. Cell viability was assessed by the activity of the cytosolic enzyme lactate dehydrogenase (LDH) liberated into the culture medium and by trypan blue exclusion. Isoproterenol and glucagon produced a significant increase of cytosolic MT about 50 %. In contrast, incubation with epinephrine and norepinephrine did not lead to any significant effects in the amount of hepatic metallothionein. Simulating the influence of cAMP by dibutyryl-cAMP (500 µM) did not affect the content of hepatic metallothionein. To examine transcriptional and translational regulatory effects supplementation of cycloheximide (0.1–500 µM) and actinomycin D (0.1–100 µM) showed a total inhibition of the agonist induced amounts. Particularly in combination with isoproterenol low LDH activities reflected a high viability of hepatocytes.

In conclusion, in primary hepatocyte cultures cAMP-mobilizing-agonists like isoproterenol and glucagon indicate an independent effect on the MT-metabolism. This is possibly due to the *de novo* synthesis of the protein because suppression by actinomycin D can be observed. However, cAMP-effectors do not seem to be involved in the induction of metallothionein because theophylline and dibutyryl-cAMP did not affect MT-metabolism by suppressing the phosphodiesterase or by stimulating the cAMP-cascade.

Received: 17 March 1995
Accepted: 12 September 1995

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Zusammenfassung Am Modell primärer Rattenhepatocytenkulturen wurde die Beteiligung von Agonisten des cAMP-Effektorsystems am Metabolismus von Metallothionein (MT) untersucht. Alle Experimente wurden in DMEM/F12 (Ham)-Medium nach 24stündiger Vorinkubation mit 5 % fetalem Kälberserum (FCS) und 20 µM Zn durchgeführt. Zur Untersuchung einer möglichen Beteiligung von cAMP-Effektoren am MT-Metabolismus wurde das Nährmedium zusätzlich mit Adrenalin (100 µM), Noradrenalin (100 µM), Glukagon (1 µM) und Isoproterenol (1–1000 µM) sowie dem synthetischen cAMP-Analogon Dibutyryl-cAMP (500 µM) versetzt. Die Konzentration an MT im Zellhomogenat wurde mittels ^{109}Cd-Bindungsassay bestimmt. Die Vitalität der Kulturen wurde über die Freisetzung der Aktivität der Laktatdehydrogenase (LDH) ins Nährmedium und der Anfärbbarkeit der Zellen mit Trypanblau nachgewiesen.

Isoproterenol und Glukagon führten zu einem signifikanten Anstieg der cytosolischen MT-Konzentration von bis zu 50 %. Im Gegensatz hierzu konnte weder nach Applikation von Adrenalin noch Noradrenalin eine signifikante Ver-

Zeitschrift für Ernährungswissenschaft, Band 34, Heft 4 (1995)

änderung der MT-Synthese beobachtet werden. Die zusätzliche Supplementierung von Theophyllin (10–1000 µM) und Propranolol (1–100 µM) als Hemmer der Phosphodiesterase bzw. von β-Rezeptoren zeigte ebenfalls keine Effekte. Die Überprüfung der regulatorischen Mechanismen auf transkriptionaler und translationaler Ebene mittels Cycloheximid (0,1–500 µM) und Actinomycin D (0,1–100 µM) ließ eine vollständige Inhibierung des durch die Agonisten provozierten MT-Anstieges erkennen. Generell niedrige LDH-Aktivitäten, vor allem in Verbindung mit Isoproterenol, waren Zeichen einer guten Vitalität des Systems.

Die vorliegende Untersuchung zeigt, daß die cAMP-Effektoragonisten Isoproterenol und Glukagon einen voneinander unabhängigen Effekt auf den Metabolismus von MT ausüben. Dabei ist der zu beobachtende MT-Anstieg auf eine *de novo* Synthese des Proteins zurückzuführen, da eine Hemmung mittels Actinomycin D möglich ist. Trotzdem muß die Beteiligung von cAMP-Effektoren am Metabolismus von MT bezweifelt werden, da weder nach Supplementierung des Phosphodiesterasehemmers Theophyllin noch des synthetischen cAMP-Analogons Dibutyryl-cAMP eine Stimulation des Systems beobachtet werden konnte.

Key words rats – hepatocytes – cAMP-cascade – metallothionein – epinephrine – norepinephrine – glucagon – isoproterenol – dibutyryl-cAMP

Schlüsselwörter Ratten – Hepatocyten – cAMP-Effektorsystem – Metallothionein – Adrenalin – Noradrenalin – Glukagon – Isoproterenol – Dibutyryl-cAMP

Abbreviation index *DMSO* = dimethyl sulfoxide · *FCS* = fetal calf serum · *HEPES* = N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid · *LDH* = lactate dehydrogenase · *MT* = metallothionein · *ns* = not significant · *p* = probability (level of significance) · *PBS* = phosphate buffered saline · *TRIS* = tris(hydroxymethyl)aminomethane · *U* = unit

Introduction

Metallothioneins were discovered in 1957, when Margoshes and Vallee (14) were searching for a tissue component responsible for the natural accumulation of cadmium in mammalian kidney. Mammalian metallothioneins (MT) are a group of polypeptides, containing 61 to 62 amino acid residues, among them 20 cysteines binding 7 equivalents of bivalent metal ions in 2 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. Metallothionein is induced *in vivo* by metals (Zn, Cu, Cd), steroids, catecholamines, polypeptide hormones and in response to acute-phases caused by various chemicals, cold, infections and starvation. For reviews see Bremner and Beattie (4) Cousins (5, 6) and Sato and Bremner (20). The mechanism of MT induction can be either a direct genomic event (metals and glucocorticoids) or an indirect, integrated physiological response to changes in neural, hormonal, or hemodynamic homeostasis. Because of this complexity, classification of various MT inducers as direct or indirect inducers may be complex when studied in intact animals. Previous *in vivo* experiments showed that rat hepatic MT is induced by agonists of the cAMP-effector-system like epinephrine and glucagon (7, 11). Use of monolayer cultures of rat liver parenchymal cells has facilitated the observation and elucidation of the mechanisms of MT induction, whereas proliferating, immortalized cell lines have changed many of the *in vivo* metabolic and structural features. Nevertheless, *in vitro* studies on the involvement of agonists of the cAMP-cascade are almost unknown. In this study the role of the cAMP-effector-system on the synthesis of MT was investigated by adding natural (epinephrine, norepinephrine and glucagon) and synthetic effectors (dibutyryl cAMP, isoproterenol and propranolol) to the medium. The model of primary rat hepatocytes was used to examine the separate influences of each mediator. The viability of the system was assessed by the integrity of cell membrane and the release of the cytosolic enzyme lactate dehydrogenase into the medium.

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 non-sterile chemicals and materials was performed by dry and wet heat (autoclaving) or for the heat sensitive collagenase enzymes and hormones through sterile filtration. Hepatocytes were isolated by a modification of the two-step collagenase perfusion procedure described by Seglen (21). 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 x 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 x 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 (22) 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 x 2 H_2O, 1.5 mM KH_2PO_4, pH 7.4). Afterwards, cells were cultured in 3 mL fresh basal medium supplemented with 10 % (v/v) FCS (7.3 µM Zn) (Myclone plus, Gibco, Eggenstein) for a further 24 h. In this 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 effectors (0.5 % of volume). The Zn concentrations of the media were approximately 20 µM. Control culture dishes were supplemented with the solvents. Experiments were performed with FCS (10 % (v/v) with 7.3 µM Zn) incorporation into the medium during the 24 h equilibration period. All incubations usually lasting 18 h were done in triplicate from two independent cell preparations (n = 3; one batch). Agonists and their concentrations tested are listed below:

Actinomycin D (SIGMA, Deisenhofen) in PBS	0.1, 1, 10, 100 µM
Butyrate (Sigma, Deisenhofen) in PBS	500 µM
Cycloheximide (SIGMA, Deisenhofen) in PBS	0.1, 1, 10, 100 µM
Dibutyryl cAMP (SIGMA, Deisenhofen) in H_2O	500 µM
Epinephrine (SIGMA, Deisenhofen) in 0.9 N NaCl	100 µM
Glucagon (SIGMA, Deisenhofen) in H_2O	1 µM
Isoproterenol (SIGMA, Deisenhofen) in 0.9 N NaCl	100 µM
Norepinephrine (SIGMA, Deisenhofen) in 0.9 N NaCl	100 µM
Propranolol (SIGMA, Deisenhofen) in 0.9 N NaCl	1, 10, 100 µM
$ZnSO_4$ x 7 H_2O (SIGMA, Deisenhofen) in H_2O	20 µM
Theophylline (SIGMA, Deisenhofen) in 0.9 N NaCl	10, 100, 500, 1000 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 phenyl-

methylsulfonyl 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 (12) 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. Cell viability was assessed by the activity of the cytosolic enzyme lactate dehydrogenase (EC 1.1.1.27, LDH) liberated into the culture medium (10). Protein concentration was evaluated according to the Lowry-Folin method (9). Media zinc was analyzed by atomic absorption spectrophotometry in an air-acetylene-flame (Philips PU 945, Kassel).

Statistical analysis

All experiments were compared by analysis of variance. After testing homogeneity of variance and if analyses indicated significant differences, treatment effects were determined by comparison to appropriate controls by LSR-test for multiple comparisons of means. Student's *t*-test was used to compare two means. Results from agonists of the cAMP-cascade were processed by regression models for elimination equations and for increase equations, evaluating a dependence on agonist concentration. Differences were considered significant if $p < 0.05$ and marked by asterisks (*).

Results and discussion

Previous time- and dose-dependent experiments detected maximal differences in metallothionein (MT)-content between control and treatment after a period of 18 h. Groups treated with isoproterenol (100 µM) showed a significant increase in cytosolic MT-content of about 30-70 %. Similar rises in MT-induction were measured in relation to 1 µM glucagon. Nevertheless, neither epinephrine (100 µM) nor norepinephrine (100 µM) led to a significant increase of hepatic MT (Fig. 1A). None of the supplements effected a significant rise in the activity of lactate dehydrogenase (LDH) released into medium. Only in the case of isoproterenol- and glucagon treatment was the viability of hepatocytes improved measured by low LDH-activities (Fig. 1A and 1B). Brady and Helvig (2) and Brady et al. (3) reported after iv- and ip-application of cAMP-mobilizing agonists significant increases in the cytosolic MT-content. In the case of the synthetic effector isoproterenol a larger amount of MT was detected than when the natural agonists epinephrine and norepinephrine were supplemented. To our knowledge no literature is available about the influence of isoproterenol on the MT-metabolism in primary hepatocytes. In the present study glucagon, epinephrine, norepinephrine and the synthetic cAMP-analogon dibutyryl-cAMP produced a significant induction of hepatic MT under similar conditions (Figs. 1A, 1B and 2). With the model of cell culture, concentrations of effectors in micromole-levels are necessary to get effects comparable with *in vivo* studies (17, 18). In previous time- and dose-dependent experiments agonist concentrations between 100 and 200 µM showed optimal increases of MT. Concentrations of 500 µM and more did not produce any higher amounts and reduced the viability of the system measured by the leakage of the cell membrane (LDH) and the condition of the cell ground. This demonstrates the narrow area of effector-concentrations necessary to get an optimal induction of MT without cytotoxical effects.

Theophylline influences the duration and intensity of the effects produced by the agonists of the cAMP-cascade by blocking phosphodiesterase and leads to a prolonged or an intensive reaction. Application of dibutyryl-cAMP simulates the effects produced by cAMP. The addition of isoproterenol (100 µM), glucagon (1 µM) and theophylline in four concentrations (10 µM, 100 µM, 500 µM and 1000 µM) was used to investigate the influence of theophylline on the induction of MT (Fig. 3A). A possible effect of theophylline on the MT-induction should be independent of the MT-induction caused by isoproterenol and glucagon. However, none of the theophylline concentrations led to a significant influence on the agonist induced MT-increase (Fig. 3A). Dibutyryl-cAMP produced only a moderate increase of cytosolic MT, which was not intensified in the presence of theophylline. However, 0.5 µM butyrate as a metabolite of the dibutyryl-cAMP catabolism caused a distinct increase of the MT-concentration (Fig. 2). In general, low LDH-activities were not influenced by adding the effectors. In groups supplemented with isoproterenol or isoproterenol plus theophylline a decrease in LDH-release was measured. Cousins and Coppen (8) also incubated primary rat hepatocytes with 0.5 mM dibutyryl-cAMP and 0.5 mM theophylline. However, a theophylline control group was missing. After 18 h incubation time a distinct increase of hepatic MT of about 50 % was observed. When both supplements were added separately or simultaneously, other authors (1) reported a 50 % or 100 % increase of cAMP respectively. Therefore the independent effect of the MT-induction by isoproterenol and glucagon, not influenced by theophylline, demonstrates that an involvement of cAMP on the MT-induction is improbable.

A clear explanation for the increasing MT-content caused by butyrate cannot be given, although comparable effects are reported in literature. In tumor cells sodium butyrate (0.5 mM) activates a number of enzymes like adenylatcyclase, but not phosphodiesterase (19). Waalkes and Wilson (23), working with an established cell line based on rat hepatocytes, reported no MT-inducing effects of cadmium when butyrate was added simultane-

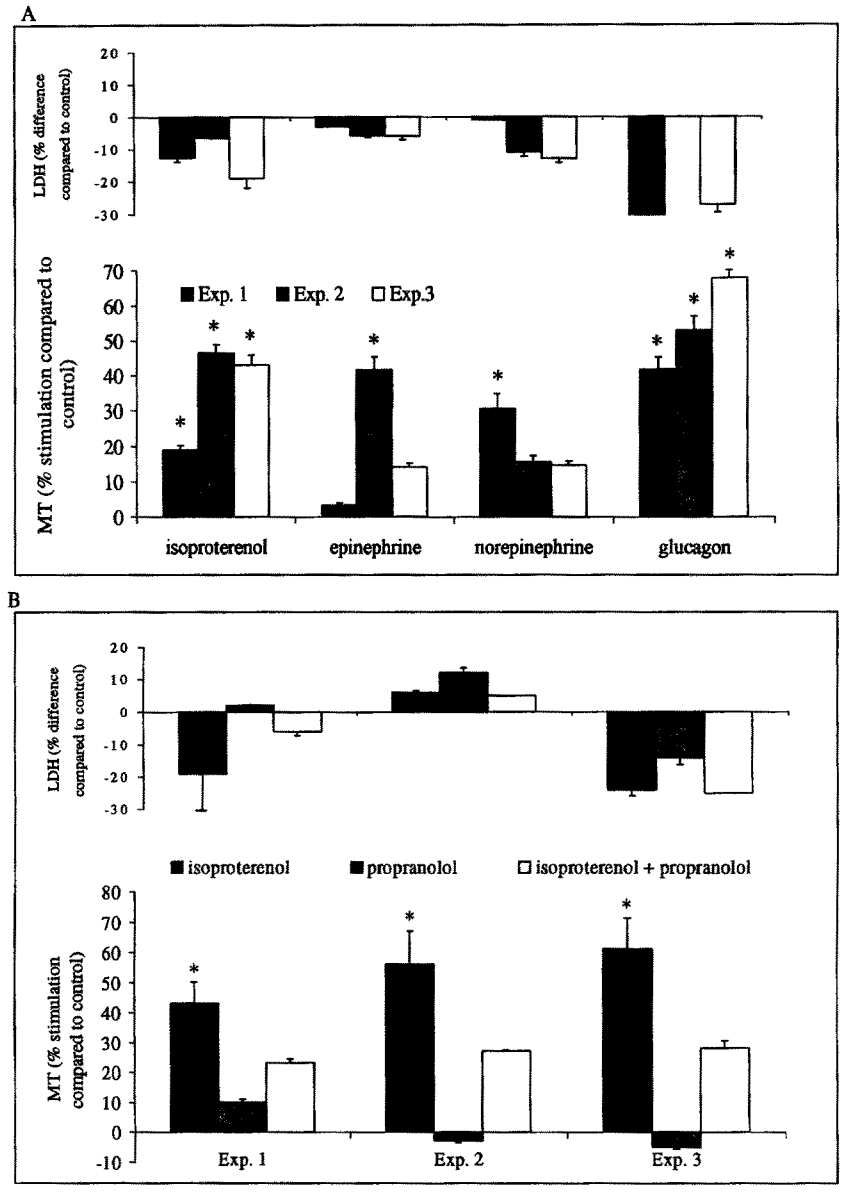

Fig. 1 Relative changes in MT-content in rat hepatocytes and activity of LDH (%) in culture media following a 18 h treatment with isoproterenol (100 µM), epinephrine (100 µM), norepinephrine (100 µM), glucagon (1 µM) (**A**), isoproterenol (100 µM) in combination with propranolol (100 µM) (**B**). Hepatocytes were cultured in DMEM/F12 (Ham) medium and preincubated for 24 h with fetal calf serum (FCS, 10 % v/v). Asterisks (*) indicate a significant difference of treatment from the representative control by LSR test ($p < 0.05$, $n = 3$).

ously. If hepatocytes were preincubated with butyrate the content of Cd-induced MT was doubled.

The ß-receptor blocking agent propranolol did not influence the basic MT-level of primary cultured rat hepatocytes in an area of 1 µM–100 µM (Fig. 3B). Simultaneous supplementation of isoproterenol (100 µM) and propranolol (1 µM) did not effect the MT-induction by isoproterenol, whereas increasing concentrations of ß-receptor blocker led to a significant reduction of the cytosolic MT-level. Equimolar application of isoproterenol and propranolol (100 µM), which show an almost identical receptor-binding-affinity, produced a 50 % inhibition of the isoproterenol-mediated MT-increase. None of the supplement combinations produced a significant change of LDH-activity in the media. Brady and Helvig (2) also describe a 50 % inhibition of the isoproterenol-(5 x 10 µg/kg LW) induced MT-increase in the liver of rats by propranolol (2 x 10 mg/kg LW). They concluded ß-receptor agonists are involved in the synthesis of MT.

On the one hand, cycloheximide, a selective inhibitor of the transketolase of 80 S-ribosomes, produces in concentrations of 0.1 µM and 1 µM a partial or complete inhibition of the isoproterenol- or glucagon induced MT-increase (Fig. 4A). On the other hand, a slight inhibition of cytosolic MT-content was measured if only 1 µM cycloheximide was supplemented. A further increase of cycloheximide concentration to 10 µM, 100 µM and 500 µM however showed no differences between control and treatment groups with isoproterenol and glucagon, whereas a dose-dependent depression of cytosolic MT-

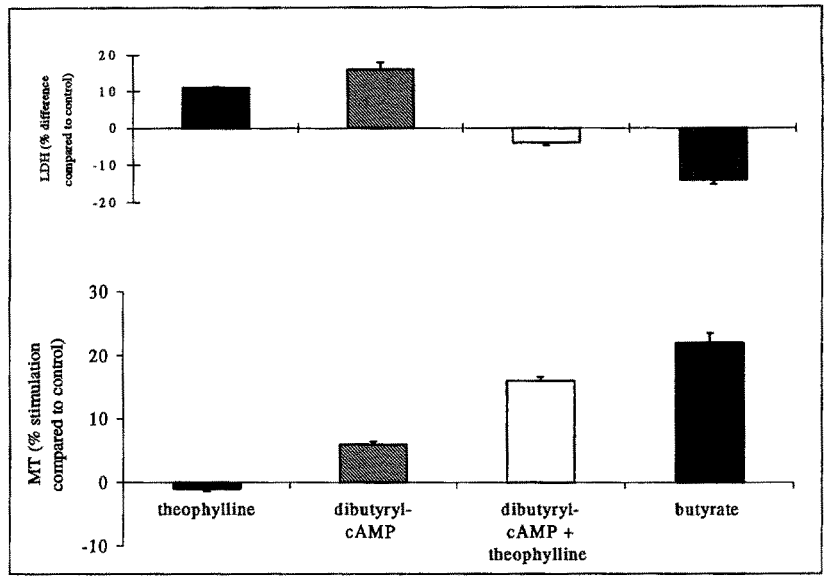

Fig. 2 Relative changes in MT-content in rat hepatocytes and activity of LDH (%) in culture media following a 18 h treatment with theophylline (500 µM), dibutyryl-cAMP (500 µM) and butyrate (500 µM). Hepatocytes were cultured in DMEM/F12 (Ham) medium and preincubated for 24 h with fetal calf serum (FCS, 10 % v/v).

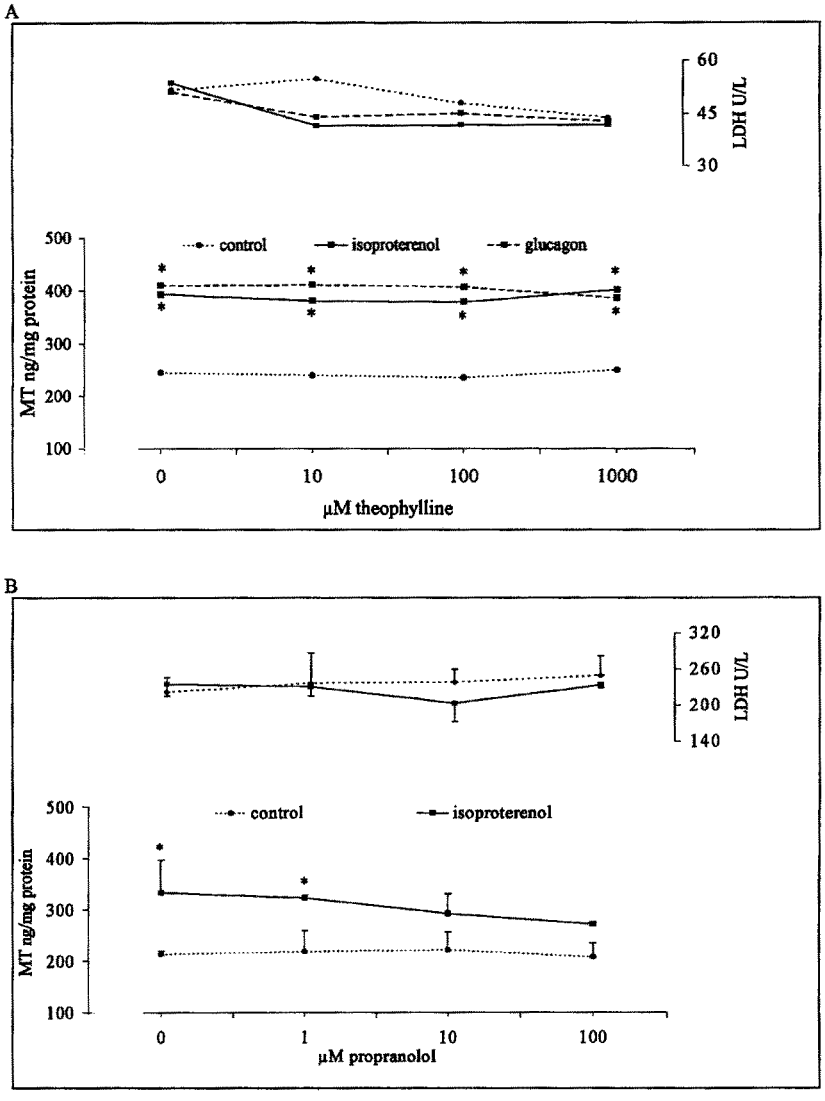

Fig. 3 Metallothionein (MT) content and activity of lactate dehydrogenase (LDH) in rat hepatocytes following a 18 h treatment with isoproterenol (100 µM) and glucagon (1µM) in combination with increasing theophylline- (10-1000 µM) (**A**) and propranolol- (1-100 µM) concentrations (**B**). Hepatocytes were cultured in DMEM/F12 (Ham) medium and preincubated for 24 h with fetal calf serum (FCS, 10 % v/v). Asterisks (*) indicate a significant difference of treatment from the representative control by LSR test ($p < 0.05$, $n = 3$).

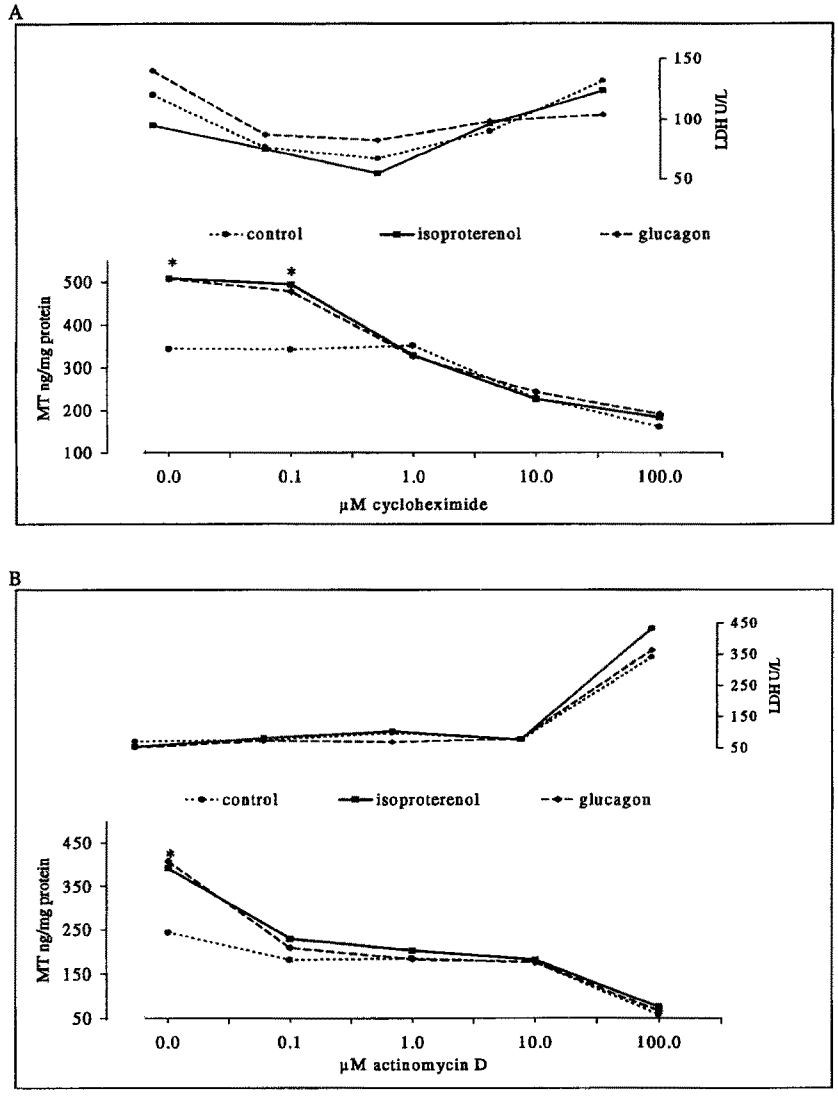

Fig. 4 Metallothionein (MT) content and activity of lactate dehydrogenase (LDH) in rat hepatocytes following a 18 h treatment with isoproterenol (100 µM) and glucagon (1µM) in combination with increasing cycloheximide- (0.1-100 µM) (**A**) and actinomycin D- (0.1-100 µM) concentrations (**B**). Hepatocytes were cultured in DMEM/F12 (Ham) medium and preincubated for 24 h with fetal calf serum (FCS, 10 % v/v). Asterisks (*) indicate a significant difference of treatment from the representative control by LSR test ($p < 0.05$, $n = 3$).

levels was measured. In a range from 0 µM to 500 µM cycloheximide supplementation the hepatic MT-content was reduced by about 40 %–70 % in groups treated with isoproterenol or glucagon and 31 %–55 % in the controls. In the case of cycloheximide supplementation, a distinct, but not significant, increase of LDH-activity was detected in all groups. Except for the hepatocytes supplemented with 500 µM cycloheximide no trypan blue stained or released cells were observed. McCormick et al. (16) reported a decrease of protein synthesis of 84 % when monolayer hepatocytes from chicken embryos were treated with 2 µM cycloheximide. Simultaneously levels of MT-mRNA rose fivefold after the addition of 8 µM - 96 µM of the transketolase-blocker, whereas a part of these MT-conserving effects is connected with a reduced turnover of MT-mRNA. Sarcoma cells of mice, also treated with cycloheximide, produced a twofold increase of MT-mRNA after a 90 min incubation period. An intensified fixing of marked uridin in the MT-mRNA points to a forced transcription (15). This can be explained by a decrease of repressor proteins and an increased rate of transcription as a consequence of the reduced protein synthesis, which can be influenced by cycloheximide reducing the catabolism of MT-mRNA (16). An explanation for these observations seems to be very complicated. Effects such as a reduced influx of Zn, a decelerated reduction of MT, an increased content of MT-mRNA and an inhibition of the protein synthesis are superimposed on each other. Studies on the influence of isoproterenol and glucagon are not reported in literature.

Actinomycin D (0.1 µM), a blocker of protein synthesis, produced a complete inhibition of the isoproterenol (100 µM)- and glucagon (1 µM)- induced MT-induction (Fig. 4B). The application of actinomycin D in concentrations of 1 µM and 10 µM led to similar results, 100 µM actinomycin D effected a significant reduction

of the hepatic MT-level. In general, none of the supplements increase the activity of LDH in the media. Only a concentration of 100 µM actinomycin D with or without isoproterenol or glucagon raised the LDH-activities to 400 U/L (Fig. 4B). Cousins and Burges (7) attributed the reduction in MT-content to a *de novo* synthesis of the protein by epinephrine (10 µM) and glucagon (1 µM) because the reaction was completely inhibited by actinomycin D. Failla and Cousins (13) reported that an accumulation of Zn led to increasing MT-levels after supplementing dexamethasone to primary hepatocyte cultures. This effect was also inhibited by actinomycin D-supplementation. A distinct decrease of glucagon-induced MT is reported in *in vivo* studies only with a concentration of 100 µM actinomycin D.

The results in literature and those in the present study indicate that the conditions for hepatocytes are suitable for studying hormonal effects proved by the established response of MT to the cAMP-effectors. This also applies to the viability measured by the release of LDH into the medium. The increase of cytosolic MT by isoproterenol and glucagon is caused by *de novo* synthesis of the protein based on the inhibitory effect of MT-synthesis through actinomycin D. The mechanism of these findings is still unclear because stimulation of cAMP-cascade with dibutyryl-cAMP and blockade of phosphodiesterase by theophylline did not influence the cytosolic MT-level. Therefore an involvement of agonists of the cAMP-effector system on the metabolism of metallothionein is questionable.

Acknowledgements This study was supported by a research grant from the Deutsche Forschungsgemeinschaft. We thank the central department of the Strahlenzentrum of the University of Giessen for giving us the opportunity to work in their laboratories.

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