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*c*AMP-mediated upregulation of gelatinases in primary cultures of isolated rat hepatocytes

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Matrix metalloproteinases (MMPs) play a major role in tissue remodelling and repair in pathophysiological conditions, such as liver fibrosis and regeneration. Regulation of the MMPs produced by liver cells is important in maintaining cell-matrix ratio in liver, which is a major target site for hormones that mediate their intracellular effects through *c*AMP. The possibility of *c*AMP affecting the activity of MMPs and their endogenous inhibitors, tissue inhibitor of MMPs (TIMPs) was studied using isolated rat hepatocytes in culture. Zymographic analysis showed that treatment with hormones like epinephrine, thyroxine and dexamethasone and Bt_2 *c*AMP increased 92 kDa MMP-9 activity. Bt_2 *c*AMP caused upregulation of MMP-9 in a dose-dependent manner. The effect of hormones was less on MMP-2. ELISA using specific antibodies showed increase in levels of MMP-9 and TIMP-1 protein. Kinetic analysis of production of MMPs and TIMPs showed that the response to Bt_2 *c*AMP was a delayed one, indicating its effect on *de novo* protein synthesis. These results suggest the possibility of *c*AMP dependent regulation of MMP-9 in the hepatocytes.

Keywords*:* Rat hepatocytes, matrix metalloproteinases (MMPs), tissue inhibitor of MMP (TIMP), cAMP-mediated upregulation, extracellular matrix (ECM), horse radish peroxidase.

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Matrix metalloproteinases (MMPs), a family of structurally-related cation-dependent neutral proteinases that are involved in degrading the components of extracellular matrix (ECM), play a major role in the tissue remodelling and repair in both physiological and pathological conditions. The abnormal expression of MMPs may contribute to pathological process, including tumour metastasis, arthritis and atherosclerosis[1]. The activity of MMPs is regulated at different levels. They are produced as proenzymes and are post-translationally activated by various agents like proteinases, oxidants and also by auto-activation. All modes of proenzyme activation lead to dissociation of cys73 from the zinc atom with concomitant exposure of active site, which is viewed as the "switch" leading to activation[2]. These enzymes are also regulated by an endogenous inhibitor known as tissue inhibitor of metalloproteinase (TIMP), which binds to the active form of MMPs, thereby blocking their activity. TIMP also inhibits the intracellular activation of zymogens. The balance between MMP and TIMP is a key determinant in the homeostasis of ECM. Transcriptional regulation of expression of MMPs is brought about by various physical and chemical signals. The physical signals, such as cell shape, heat shock, cytochalasin B treatment, and adhesive interaction of the cells with matrix proteins like fibronectin and laminin induce MMP gene expression[3]. The chemical signals like cytokines, growth factors[4-6] and hormones[7-9] produce variable effects in different systems. The hormonal modulation of MMPs plays an important role in matrix remodelling during involution of mammary gland[10] and uterus, neovascularisation of cornea, and bone resorption[3].

The ECM also plays a critical role in the regulation of hepatic function[11], although the relative proportion of connective tissue to parenchyma is very small in the liver. Under pathological conditions, such as liver fibrosis, there is an accumulation of ECM. In hepatic regeneration, remodelling of ECM occurs. Apart from the regulation of the synthesis of matrix components,

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Abbreviations: MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; ECM, extracellular matrix; Bt_2 *c*AMP, dibutyryl cyclic adenosine monophosphate; HRP, horse radish peroxidase.

regulation of the molecular mechanisms involved in their degradation also appears to be critical in the maintenance of ECM homeostasis in liver[11]. The degradative process involves MMPs that are produced by the liver cells and the regulation of MMPs is therefore important in the maintenance of cell-matrix ratio in the liver. The liver is a major target site of hormones, which generally mediate their intracellular effects through *c*AMP. In the present paper, possibility of *c*AMP affecting the activity of MMPs and their endogenous inhibitors TIMPs in liver was studied using isolated rat liver cells in culture and the results are presented here.

Materials and Methods

Eagle's MEM, type IV collagenase, penicillin, streptomycin, gelatin, dibutyryl *c*AMP (Bt_2 *c*AMP), *o*-dianizidine, antibodies against MMP-2 MMP-9, TIMP-1 and TIMP-2, nitrocellulose membrane, antirabbit IgG-HRP, acrylamide, bisacrylamide, thyroxine, epinephrine and dexamethasone were from Sigma (St. Louis, MO, USA). All other chemicals were analytical grade reagents. Plastic tissue culture dishes were purchased from NUNC (Denmark).

Isolation and culture of hepatocytes

Hepatocytes were isolated from the liver of rats (Sprague-Dawley strain) by collagenase perfusion[12]. The viability of the cells was tested by trypan blue exclusion and only cell preparations with more than 90% viability were used for experiments and they retained their viability in culture. Hepatocytes in minimum essential medium (MEM) were seeded on 35 mm plastic dishes, passively coated with col I [50 μg/ml] and after 4 hr, unattached cells were removed and the attached cells were incubated with medium containing Bt_2 *c*AMP. After the required time intervals, the medium was collected and the cells were harvested. Medium was used for zymography and ELISA. The cell protein was estimated by the method of Lowry *et al*[13].

Zymography

The activity of MMPs was determined by zymography[14]. Samples equivalent to same amount of cell protein were subjected to electrophoresis in substrate gel. Zymogram gels consisted of 7.5% polyacrylamide gel copolymerized with gelatin (1 mg/ml). After electrophoresis, gels were washed twice for 30 min each in 2.5% Triton X-100 at room temperature, incubated for 24-48 hr in substrate buffer (50 m*M* Tris-HCl, 5 m*M* $CaCl_2$, 0.02% NaN_3, *p*H 7.5) at 37°C and were stained with Coomassie blue R-250 and destained with water. Gelatinase activities were seen as clear bands. The gels were scanned in a Biorad gel documentation instrument and intensity of bands was quantitated by Quantity One – 4.5.0 – software (Biorad).

ELISA

Multi well plates were coated with culture medium and incubated with antibodies against MMP-2 and MMP-9 for 2 hr. Anti-MMP-2 (catalogue no. 6302 amino terminal end) developed in rabbit reacts with bands at 72 kDa and 68 kDa; Anti-MMP-9 (catalogue no. 5177 carboxy terminal end) developed in rabbit reacts with bands at 92 kDa and 88 kDa. It was then washed with PBS-Tween, treated with secondary antibody conjugated to horse raddish peroxidase (HRP) and the colour was developed using *o*-dianisidine[15].

SDS-PAGE and Western blotting

SDS-PAGE was done according to the procedure of Laemmli[16]. Western blotting was done[17] using a semidry apparatus.

Results and Discussion

Production of MMPs by isolated rat hepatocytes in culture

Zymographic analysis of the culture medium of hepatocytes showed the presence of three major bands, which migrated at M(r) 130 kDa, 92 kDa/88 kDa and 72 kDa/68 kDa. Among these, 92 kDa/88 kDa band was identified as gelatinase B (MMP-9) and 72 kDa/68 kDa as gelatinase A (MMP-2) by immunoblot analysis using specific antibodies (Fig. 1).

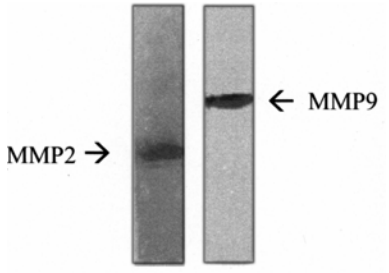

Fig. 1—Immunoblot analysis of MMP-2 and MMP-9 [Isolated hepatocytes were maintained in culture for 24 hr and the medium was subjected to SDS-PAGE and Western blotting. The proteins were probed against MMP-2 and MMP-9, followed by peroxidase conjugated IgG. Colour was developed using 4-chloro-1-naphthol reagent]

Effects of hormones on MMP production by primary cultures of rat hepatocytes

The effect of hormones on MMP production by hepatocytes was studied by treating cells in culture with different hormones [thyroxine (10^{-7} *M*), epinephrine (10^{-5} *M*) and dexamethasone (10^{-4} *M*)] for 18 hr. The medium was collected and MMP activity was measured by zymography and by ELISA, using specific antibodies against MMP-9 and MMP-2. All the three hormones at the concentration tested caused increase in activity of MMP-9, but in the case of MMP-2, only epinephrine showed an increase in activity, while thyroxine and dexamethasone had no significant effect (Fig. 2A). ELISA showed a significant increase in production of MMP-9 protein, on treatment with the three hormones. But, only epinephrine caused a significant increase in MMP-2 protein level, while thyroxine and dexamethasone showed no significant change in the production of MMP-2 (Fig. 2B).

Effect of Bt_2 *c*AMP on MMP production by primary cultures of rat hepatocytes

The effect of Bt_2 *c*AMP was studied by treating hepatocytes in culture with different concentrations (10^{-6}-10^{-3} *M*) of Bt_2 *c*AMP. The cultures were maintained for 18 hr after which the medium was collected and gelatinase activity was measured by zymography and succinylated gelatin assay (data not given). There was increase in the activity of MMP-9 with increase in concentration of Bt_2 *c*AMP, but the effect was less on MMP-2. At a concentration of 10^{-3} *M* , a significant increase in the activated form of MMP-9 was observed, but no such activation was observed in the case of MMP-2 (Fig. 3A).

Bt_2 *c*AMP dependent effect on the production of gelatinases was further investigated by treating the cells for different time intervals (4-24 hr) with Bt_2 *c*AMP (10^{-4} *M*). During the earlier time intervals, no significant effect on gelatinase activity was detected.

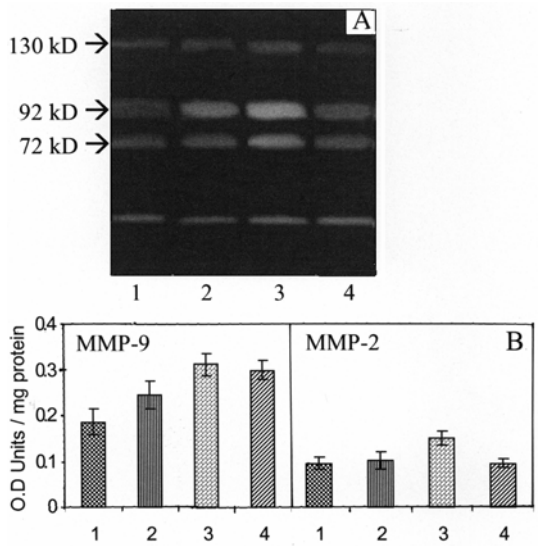

Fig. 2—Production of MMPs by hepatocytes: Effect of hormones - zymography [Isolated hepatocytes were maintained in culture in the presence of different hormones [Lane 2, thyroxine (10^{-7} *M*), lane 3, epinephrine (10^{-5} *M*) and lane 4 dexamethasone (10^{-4} *M*)] for 18 hr. The medium was collected and protein equivalent volumes were used in **zymography** (A) and in **ELISA** (B) using specific antibodies against MMP-9 and MMP-2; Untreated cells served as control, lane 1]

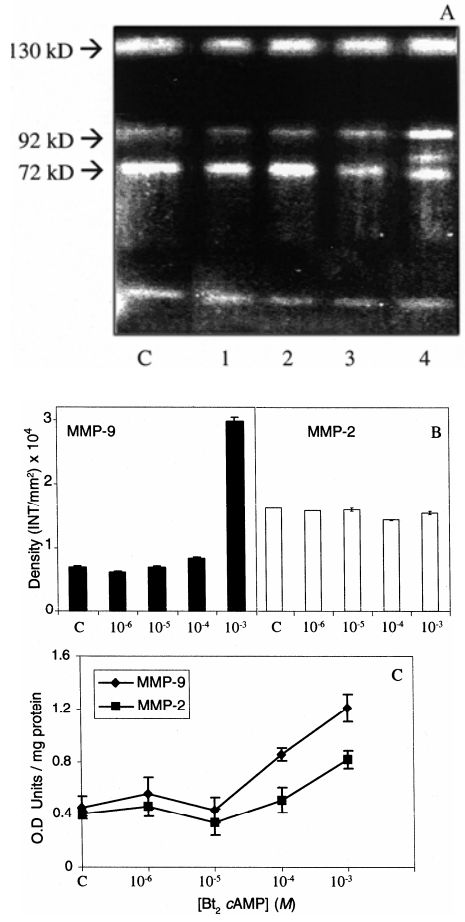

Fig. 3—Production of MMPs by hepatocytes: Effect of Bt_2 *c*AMP-concentration dependence [Isolated hepatocytes were maintained in culture in presence of different concentrations of Bt_2 *c*AMP [10^{-6} (1), 10^{-5} (2), 10^{-4} (3) and 10^{-3} (4) *M*] for 18 hr. The medium was collected and protein equivalent volumes were used in **zymography** (A), activity was measured by scanning the bands and quantified the intensity using programme Quantity One and expressed as intensity units/mm^2 (B), MMPs were also analysed by **ELISA** (C) using specific antibodies against MMP-9 and MMP-2]

But, at later time intervals increase in the activity of MMP-2 and MMP-9 was observed; maximum effect on MMP-9 was observed at 24 hr in zymography (Fig. 4A).

The effect of Bt_2 *c*AMP on MMP production was further analysed by ELISA using specific antibodies against MMP-2 and MMP-9 and the results are shown in Fig. 3C. An increase in the production of MMP-2 and MMP-9 with increase in concentration of Bt_2 *c*AMP was observed. The effect of Bt_2 *c*AMP was more in the case of MMP-9. Although there was no significant increase in the amount of MMPs on treatment with Bt_2 *c*AMP during the earlier time intervals, ELISA showed a significant increase in the amount of MMP-9 protein at 24 hr (Fig. 4C).

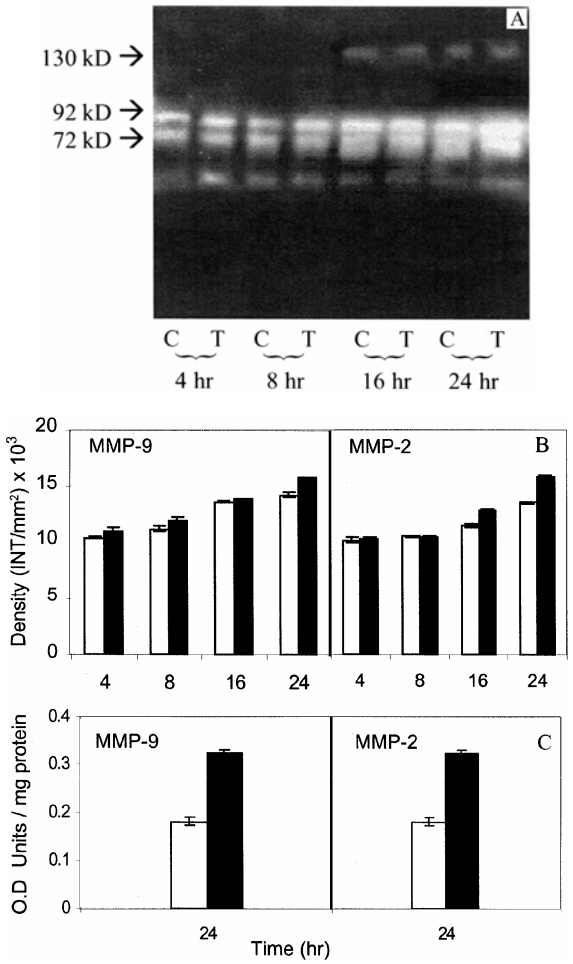

Fig. 4—Production of MMPs by hepatocytes: Effect of Bt_2 *c*AMP-time dependence [Isolated hepatocytes were maintained in the presence of 10^{-4} *M* Bt_2 *c*AMP for different time intervals (4-24 hr). The medium was collected and protein equivalent volumes were used in **zymography** (A), activity was measured by scanning the bands and quantified the intensity using programme Quantity One and expressed as intensity units/mm^2 (B), MMPs were also analysed by ELISA (C) using specific antibodies against MMP-2 and MMP-9 and compared with control (□). Bt_2 *c*AMP (■)]

Effect of Bt_2 cAMP on production of TIMP in primary cultures of hepatocytes

The effect of Bt_2 *c*AMP on production of TIMP was studied by treating hepatocytes in culture with different concentrations (10^{-6}-10^{-3} *M*) of Bt_2 *c*AMP. The cultures were maintained for 24 hr after which the medium was collected and the amount of TIMP present in the medium was determined by ELISA using specific antibodies against TIMP-1 and TIMP-2, and the results are shown in Fig. 5A. At lower concentrations of Bt_2 *c*AMP, no significant change in the amount of TIMPs was observed. But, at a concentration of 10^{-4} *M*, a significant increase in the amount of TIMP-1 and TIMP-2 was observed. However, further increase in concentration to 10^{-3} *M* caused a decrease in the level of TIMP.

Bt_2 *c*AMP dependent effect on the production of TIMPs was further investigated by treating the cells for different time intervals (4-24 hr) with Bt_2 *c*AMP

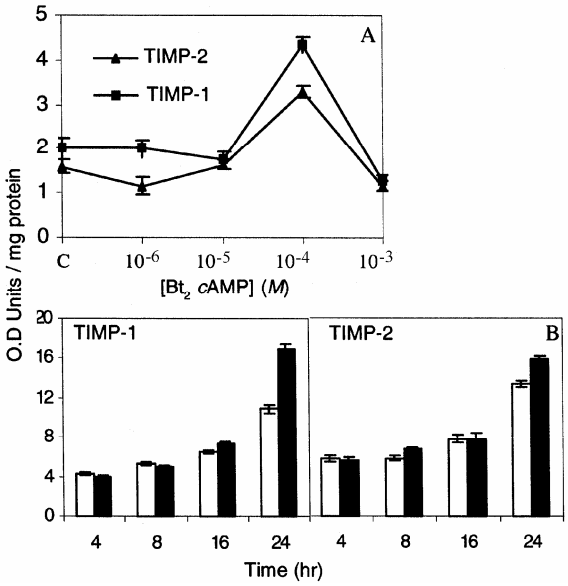

Fig. 5—Production of TIMPs by hepatocytes (A): Effect of Bt_2 *c*AMP-concentration dependence [Isolated hepatocytes were maintained in presence of different concentrations of Bt_2 *c*AMP [10^{-6} -10^{-3} *M*] for 24 hr. The medium was collected and protein equivalent volumes were analysed by **ELISA** using specific antibodies against TIMP-1 and TIMP-2]; and (B): Effect of Bt_2 *c*AMP - time dependence [Isolated hepatocytes were maintained in presence of 10^{-4} *M* Bt_2 *c*AMP for different time intervals (4-24 hr). The medium was collected and protein equivalent volumes were analysed by **ELISA** using specific antibodies against TIMP-1 and TIMP-2 and compared with control (□). Bt_2 *c*AMP (■)]

(10^{-4} *M*). During the earlier time intervals, no significant effect on TIMP was detected. ELISA showed a significant increase in the amount of TIMP-1. The effect of Bt_2 *c*AMP was more on TIMP-1 than on TIMP-2 at 24 hr (Fig. 5B).

Although ELISA showed increase in the level of MMP-protein, that much increase in activity on zymogram could not be found, particularly in the case of MMP-9, possibly due to the corresponding increase in its TIMP level in response to Bt_2 *c*AMP or due to variation in the extent of activation of proenzyme.

The above results indicate that hormones and substance like Bt_2 *c*AMP which alter intracellular levels of *c*AMP caused alteration in MMP-9 activity in the hepatocytes. Further kinetic analysis of the production of gelatinase showed that the effect of Bt_2 *c*AMP is maximum at 24 hr and indicated the delayed nature of response. Bt_2 *c*AMP upregulates MMP-9 synthesis in monocyte[6], whereas elevation of intracellular *c*AMP was reported to inhibit MMP-9 induction in keratinocytes[18], showing its cell-specific nature of regulation. Bt_2 *c*AMP is also reported to have no effect on the expression of MMP-2 and MMP-9 in human breast epithelial cancer cells[19], but switches MMP-TIMP balance in favour of TIMP-2, which is transcriptionally regulated at high levels of Bt_2 *c*AMP. Thus, the above reports indicate that the effect of Bt_2 *c*AMP varies with the cell type. But, in our system, MMPs and TIMPs levels are upregulated by Bt_2 *c*AMP, which appears to be physiologically important, since the relative proportion of ECM in the liver is very low. Transcriptional regulation of TIMP-2 by Bt_2 *c*AMP at high concentration[19] suggested that the *c*AMP effect may be a protein kinase A independent effect. The absence of effects of Bt_2 *c*AMP at low concentrations on MMP production in hepatocytes indicates the possibility of involvement of a similar protein kinase A independent mechanism.

In liver fibrosis, normal cell-matrix interactions are disturbed due to the remodelling or degradation of matrix, contributed mainly by MMPs. In human liver disease and in animal models of liver fibrosis, there is evidence of increased expression of gelatinase A[20,21] and gelatinase B[22]. MMP-2 and MMP-9 are also found to be increased in liver regeneration after partial hepatectomy[23,24]. Our results showed that *c*AMP mediates the upregulation of MMP-9 in rat hepatocytes. It is thus possible that *c*AMP may also contribute to the increased expression of gelatinases in events such as liver fibrosis and regeneration, and drugs that modulate the production of *c*AMP may influence cell-matrix ratio in the liver.

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