Human Metallothionein Gene Expression Is Upregulated by β-Thujaplicin: Possible Involvement of Protein Kinase C and Reactive Oxygen Species

Hajime Nakano,* a Satsuki Ikemaga, a Takayuki Aizu, a Takahide Kaneko, a Yasushi Matsuzaki, a
Shigeki Tsuchida, b Katsumi Hanada, a and Yaeno Arima a

*Department of Dermatology, Hirosaki University School of Medicine; b Second Department of Biochemistry, Hirosaki University School of Medicine; Hirosaki 036–8562, Japan; and c Department of Dermatology, Kyoto University Graduate School of Medicine; Kyoto 606–8501, Japan.

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Recently, we discovered that β-thujaplicin (BT) induces metallothionein (MT) expression in mouse keratinocytes, both in vivo and in vitro. However, the molecular mechanisms by which BT exerts its biological effects have not been elucidated. The purpose of this study is to explore the signal transduction pathway involved in the MT mRNA induction by BT. Using a HaCaT keratinocyte cell line, Northern blotting was performed for analyzing the human MT-IIA mRNA expression levels in combination with BT and a number of protein kinase (PK) inhibitors including H7, HA1004 and a PKC-specific inhibitor chelerythrin. CAT assays with the MT-IIA gene promoter-CAT construct were conducted for examining the transcriptional regulation by BT of MT. A free radical scavenger N-acetylcysteine (NAC) was used for analyzing a role of oxidative stress for the MT gene induction by BT. BT increased MT-IIA gene transcript levels and CAT activity in a dose-dependent fashion in HaCaT cells. The increase in MT-IIA mRNA levels and CAT activity were completely suppressed by H7 but not by HA1004. In addition, chelerythrin prevented BT-inducible MT-IIA promoter activation. Furthermore, NAC suppressed BT-inducible MT-IIA promoter activation. These results demonstrate that BT is a potent activator of the MT-IIA gene promoter and that PKC activation and reactive oxygen species are implicated in BT-inducible MT-IIA gene expression. BT may be a useful tool for dissecting the signal transduction pathway mediating MT-IIA promoter activation.

Key words β-thujaplicin; metallothionein; gene regulation; protein kinase C; reactive oxygen species

β-Thujaplicin (BT), also known as hinokitiol, is a tropolone-related compound found in the heart wood in several cupressaceous plants such as the Hinoki cypress, Chamaecyparis obtusa, and the western red cedar, Thuja Plicata Don. BT has a wide variety of biological functions, including antibacterial and antifungal activity, acting as an iron chelator for Salmonella typhimurium, anti-tumor activity, and induction of differentiation. Thus far, however, the molecular mechanisms by which BT exerts such a broad spectrum of biological effects have not been elucidated.

Metallothioneins (MT) are ubiquitously distributed, low molecular weight proteins with a high cysteine content and high binding capacity for metals such as zinc and cadmium. MT function in zinc homeostasis and detoxification of heavy metals. Several experiments have also shown that MT acts as a reactive oxygen species (ROS) scavenger, and MT induction has protective effects against oxidative stresses such as anticancer drugs and ultraviolet (UV). MT gene expression is induced not only by heavy metals but also by a wide variety of stress-inducing agents such as ultraviolet, X-ray, hypoxia, hydrogen peroxide, and a number of cytokines including interferon-α and β, TNF-α, and interleukin 1 and 6. Recently, we found that BT induces cellular metallothionein in mouse keratinocytes in vitro and in vivo, resulting in reduction of apoptotic cell formation caused by UV-B irradiation.

The molecular mechanism underlying MT gene regulation has been extensively investigated (reviewed in ref. 24). In the proximal promoters of MT genes, multiple copies of metal response elements (MREs) reside, and these cis-elements are essential for MT gene induction by zinc and cadmium. MREs were shown to mediate the transcriptional response of MT genes to oxidative stress in mouse hepatoma cells. A metal-responsive transcription factor, termed MTF-1, which binds to MREs and activates MT gene transcription, has been cloned from mouse and human. Recently, the involvement of protein kinase-C (PKC) has been demonstrated in zinc- and cadmium-induced MT gene expression, as determined by Northern analyses using a specific PKC inhibitor, chelerythrin. However, the precise regulation of MT gene expression by other inducing agents has yet to be elucidated.

In the present study, we report the first evidence for BT as a potent transactivator for the human MT-IIA gene. Moreover, inhibition experiments revealed a possible involvement of protein kinase C and reactive oxygen species in the transduction mechanism.

MATERIALS AND METHODS

Chemicals BT was a kind gift from Takasago (Tokyo, Japan). Cycloheximide, H7, chelerythrin, HA1004, and N-acetylcysteine (NAC) were all purchased from Sigma (St. Louis, MO, U.S.A.).

Cell Culture The spontaneously transformed human epidermal keratinocyte cell line, HaCaT, (kindly provided by Dr. Husenig) was cultured in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 1% l-glutamine, and 1% antibiotic/antimycotic solution. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. The viability of the cells treated with 250 μM BT for 24 h was approximately 70% as...
determined by trypan blue exclusion staining.

Northern Blot Analysis The procedure for Northern blot analysis is described elsewhere. Blots were probed with a 260-bp fragment of the human MT-IIA cDNA generated by RT-PCR, labeled by the random primer method. Primers for RT-PCR were designed from the published MT-IIA gene sequence. Hybridization signals were quantified by densitometry and normalized to 7S RNA levels.

Plasmid Constructs The vector p5′MT-CAT, containing the 5′-flanking region of the MT-IIA gene, was generated by ligating a HindIII/BamHI fragment of the MT-IIA promoter, spanning from −764 to +79, (American Type Culture Collection, Rockville, MD, U.S.A.) to the pBS0CAT reporter construct. The human type VII collagen gene (COL7A1) and the human BPAG1 promoter/reporter constructs were described elsewhere. The integrity of the reporter constructs was confirmed by direct sequencing.

Transient Transfection Experiments and Reporter Assays Transient transfection was performed using TransIT Transfection Reagent (PANVERA, Madison, WI, U.S.A.). Briefly, 60% confluent HaCaT cells in a 60 mm dish were incubated with 0.5 μg reporter gene and transfection reagent for 6 h. For monitoring transfection efficiency, cells were cotransfected with the RSV-β-galactosidase expression vector. Cells were washed 3 times with PBS, followed by further incubation with DMEM containing 0.1% FCS overnight (12 h). After additional incubation with or without BT for 6 h, cells were rinsed twice with PBS and then lysed in 200 μl of Reporter Lysis Buffer (Promega, Madison, WI, U.S.A.). CAT assays were performed as described previously.

RESULTS

Induction of MT-IIA mRNA by BT We previously demonstrated that mouse MT-I mRNA levels are induced by BT in the transformed mouse keratinocyte cell line, Pam 212. Figure 1a shows that in human HaCaT keratinocyte cells, BT increased the amount of MT-IIA mRNA transcripts in a dose-dependent manner. Time-course analysis showed that the increase in MT-IIA mRNA occurred as early as 1 h after treatment, with a maximal induction at 6 h (Fig. 1b).

No Requirement for Ongoing Protein Synthesis for BT-Induced MT-IIA Gene Expression To examine whether the BT-induced increase in MT-IIA gene expression was dependent on ongoing protein synthesis, we performed similar induction experiments in the presence or absence of the protein synthesis inhibitor, cycloheximide. As shown in Fig. 2, 10 μg/ml of cycloheximide treatment did not inhibit BT-induced MT-IIA gene expression. These findings indicate that MT-IIA mRNA induction does not require ongoing protein synthesis.

Evidence for Transcriptional Upregulation of MT-IIA by BT To examine whether the BT-induced increase in MT-IIA mRNA levels is regulated at the transcriptional level, HaCaT cells were transiently transfected with a CAT reporter construct bearing the MT-IIA gene promoter region. Promoter activity was induced in a dose-dependent manner by the addition of increasing amounts of BT to the culture media. Cultures incubated with 250 μM BT for 6 h showed a 40-fold induction in promoter activity relative to control cultures without BT (Fig. 3a). At the same time, 250 μM BT had no effect on CAT activity in cells transfected with a thymidine kinase promoter-CAT construct (Fig. 3b), indicating that BT did not alter the stability of the CAT mRNA transcript or CAT protein. These results suggest that the induction of MT-IIA mRNA levels by BT occurs at the transcriptional level. In addition, BT failed to stimulate a number of other reporter constructs, including human COL7A1 (Fig. 3b) and human BPAG1 promoter-CAT constructs (data not shown), which have well characterized promoter functions in
cultured human keratinocytes.29,32) These findings imply that the effects of BT on the MT-IIA promoter are specific.

Involvement of Protein Kinase C in BT-Activated Signal Transduction

To explore the potential molecular mechanisms responsible for BT-inducible MT-IIA gene expression, a general protein kinase inhibitor, H7, was applied to HaCaT cells 2 h before administering BT. As indicated in Fig. 4a, Northern blotting showed that BT failed to induce MT-IIA mRNA expression in the presence of H7. Transient transfection using the MT-IIA promoter-CAT construct demonstrated that H7 effectively prevented MT-IIA promoter activation elicited by BT (Fig. 4b). Since H7 inhibits not only PKC but also PKA, we next utilized chelethrin, a specific PKC inhibitor. As shown in Fig. 4c, BT-induced MT-IIA promoter activation was inhibited by chelethrin in a dose-dependent manner. We then treated HaCaT cells with a PKA-specific inhibitor, HA1004, to examine whether PKA is involved in MT-IIA promoter activation elicited by BT. Figure 4d showed that HA1004 had little effect on BT-induced MT-IIA promoter activity.

A Possible Role for Reactive Oxygen Species in MT-IIA Promoter Activation by BT

Oxidative agents, such as ultraviolet14) and hydrogen peroxide,17) are capable of inducing MT gene expression in various cell lines. We therefore performed a CAT reporter assay, using the MT-IIA promoter-CAT construct, in combination with the free radical scavenger N-acetylcysteine (NAC), a glutathione precursor. Thirty mM NAC by itself did not affect the basal activity of the MT-IIA gene promoter (Fig. 5). BT-induced promoter activation was strongly inhibited by the presence of NAC at a concentration as low as 5 mM, suggesting the possible involvement of reactive oxygen species in MT-IIA promoter activation by BT.

DISCUSSION

In this study, we demonstrated that BT significantly induces MT-IIA mRNA expression in the spontaneously transformed human keratinocyte cell line, HaCaT. Ongoing protein synthesis was not required for BT-induced MT-IIA mRNA expression (Fig. 2), consistent with the relatively rapid kinetics of MT mRNA induction shown in Fig. 1b. Such an induction profile resembles that of heavy metals and hydrogen peroxide.17) These data suggest that pre-existing molecule(s) in the signal transduction pathway may be utilized for upregulation of MT-IIA gene expression upon stimulation by BT.

To examine whether the increased steady-state MT-IIA mRNA levels are induced by BT at the transcriptional level,
transient transfection experiments and CAT assays using the MT-IIA promoter-CAT reporter construct were performed using HaCaT cells. The results demonstrated that BT could effectively and specifically transactivate the MT-IIA gene promoter (Figs. 3a, b). To further explore the signal transduction mechanism involved in BT-induced MT-IIA gene expression, we conducted inhibition experiments using a number of protein kinase inhibitors. A general protein kinase inhibitor, H7, significantly prevented BT-inducible MT-IIA mRNA expression, as well as transactivation of the MT-IIA promoter (Figs. 4a, b). In addition, a PKC-specific inhibitor, chelerythrin, abolished BT-inducible promoter activity in a dose-dependent manner, whereas a PKA-specific inhibitor HA1004 failed to inhibit promoter activity (Figs. 4c, d). These data strongly suggest that PKC is involved in the transduction pathways mediating the induction of MT-IIA gene expression by BT in the cell line examined.

In the present study, we demonstrated that NAC, a glutathione precursor completely inhibited BT-inducible MT-IIA promoter activation in a dose-dependent manner (Fig. 5), implying that reactive oxygen species may be involved in the upregulation of MT-IIA gene expression by BT. MT gene expression is known to be induced by agents capable of producing reactive oxygen species, such as hydrogen peroxide, paraquat, and UV. Recently, the DNA binding activity of the metal-responsive transcription factor MTF-1 in mouse Hepa cells was shown to be enhanced not only by heavy metals, but also by hydrogen peroxide. Furthermore, LaRochelle et al. revealed that recombinant MTF-1 was phosphorylated upon stimulation with zinc chloride in vivo, and co-expression of a dominant-negative PKC mutant inhibited metal-induced and MTF-1-dependent promoter activation, indicating that phosphorylation is involved in MTF-1 activation in response to metal ions. These results led us to hypothesize that BT produces reactive oxygen species (ROS) in reaction with cellular macromolecule(s), thereby activating MTF-1 via PKC, resulting in the transcriptional upregulation of the MT-IIA gene. Palmiter demonstrated that pyrrolidine dithiocarbamate (PDTC), a metal chelator, was capable of inducing MT gene expression and stimulating the MTF-1-dependent promoter, and proposed that PDTC probably acts by increasing metal transport into cells. As BT has been demonstrated to be a lipophilic chelator, which can chelate various heavy metal ions, including iron and zinc, it may act as a...
transporter of extracellular heavy metals. Intracellularly-delivered metal ions by BT may then generate ROS such as hydroxyl radical, superoxide radical or hydrogen peroxide, resulting in the MTF-1 activation and enhancement of MT gene transcription. Another possible mechanism of ROS production by BT is that this compound may directly affect cellular organelle(s) e.g. mitochondria, leading to ROS generation. Indeed, Nakagawa and Tayama have demonstrated that the cytotoxic effects of BT on freshly isolated rat hepatocytes are associated with mitochondria dysfunction. It would be important to note that Dp44mT, a lipophilic Fe chelator, has been demonstrated to cause apoptosis in association with single oxygen, hydrogen peroxide, or hydroxyl radical in the murine Madison-109 lung cancer cells. Further studies are now in progress to clarify whether MTF-1 is involved in the inducible transcriptional upregulation of the MT-IIA gene by BT.

Another candidate target molecule for mediating the effects of BT may be the activator protein-1 (AP-1), since AP-1 is stimulated by a number of PKC-activating agents, such as a tumor promoter 12-O-tetradecanoylphorbol-13-acetate and UV. Moreover, this activation results in induction of MT-IIA gene expression. However, BT failed to stimulate the COL7A1 promoter (Fig. 3b), which has been shown to be significantly activated by UV irradiation in association with the induction of AP-1 binding activity, and a mutation in the functional AP-1 binding site remarkably abolished the UV-inducible enhancement in COL7A1 promoter activity. On the other hand, Yu et al. demonstrated that chelerythrin inactivated zinc- and cadmium-inducible metallothionein gene expression in cadmium resistant, metallothionein gene amplified Chinese hamster ovary cells, whereas a deletion mutant of the mouse MT-I promoter lacking the AP-1 binding site was still stimulated by zinc and cadmium. Thus, further study is needed to clarify whether BT can induce AP-1 transcriptional activity, as well as whether AP-1 activation is required for BT-inducible MT-IIA gene expression.

In conclusion, we report that BT is a potent activator of the MT-IIA gene promoter and that PKC activation and reactive oxygen species are implicated in BT-inducible MT-IIA gene expression. BT may be a useful tool for dissecting the signal transduction pathway mediating MT-IIA promoter activation.

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