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Original Contribution

BMP6 attenuates oxidant injury in HK-2 cells via Smad-dependent HO-1 induction

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ABSTRACT

Oxidative stress is involved in a variety of kidney diseases, and heme oxygenase 1 (HO-1) induction is a protective response to oxidative stress. Downregulation of bone morphogenetic protein 6 (BMP6) is associated with renal damage in intrauterine growth-restricted newborns. However, it is unknown whether BMP6 has a renoprotective effect or HO-1 induction property. In this study, we demonstrate that BMP6 effectively protects renal proximal tubule cells (HK-2) against hydrogen peroxide (H_2O_2)-induced cell injury. BMP6 also increased HO-1 gene expression and activity of HO. Inhibition of de novo gene expression, the HO inhibitor ZnPPIX, HO-1 knockdown, or the carbon monoxide (CO) scavenger hemoglobin attenuated the cytoprotective effect of BMP6, whereas HO-1 constitutive expression, the HO-1 inducer hemin, or the hemin metabolites bilirubin and CO ameliorated H_2O_2-induced cell injury. Stimulation of HK-2 cells with BMP6 activated Smad signaling but not mitogen-activated protein kinases. In addition, BMP6-mediated induction of HO-1 expression and increase in HO activity were inhibited by Smad5 knockdown. Furthermore, deletion or mutation of the Smad-binding element in the HO-1 promoter also inhibited BMP6-induced luciferase activity. In summary, these findings suggest that induction of HO-1 through a Smad-dependent mechanism is responsible for the cytoprotective effect of BMP6 in H_2O_2-mediated renal cell injury.

Increased oxidative stress and high-level reactive oxygen species (ROS)[1]-mediated cell damage have been implicated in a variety of kidney diseases, such as renal ischemia–reperfusion injury [1], diabetic nephropathy [2], and chronic tubulointerstitial injury [3]. ROS formation is a naturally occurring process. Mammalian cells have developed several protective mechanisms to prevent ROS formation or detoxify ROS. These mechanisms employ low-molecular-mass antioxidants (ascorbic acid, glutathione, tocopherols, and others), ROS-interacting enzymes (superoxide dismutase, peroxidases, and catalases), and redox regulation enzymes [4]. Among the various protective enzymes, extensive studies suggest that heme oxygenase 1 (HO-1) plays a central role in maintaining antioxidant homeostasis during cellular and tissue oxidative stress [5,6].

Heme oxygenase is a microsomal enzyme. There are at least two well-characterized isoenzymes, inducible HO-1 and constitutive HO-2. HO-1, also known as HSP32 (heat-shock protein of 32 kDa), is induced by a wide variety of stimuli that include heme products, hydrogen peroxide, ultraviolet A radiation, heavy metals, endotoxin, cytokines, and oxidative stress [7]. HO-1 catalyzes the degradation of heme to release free iron, carbon monoxide (CO), and biliverdin. Biliverdin is subsequently converted to bilirubin by the enzyme biliverdin reductase. Recent reports indicate that these products possess potent antioxidant, vasodilatory, antiapoptotic, and anti-inflammatory properties [8–12]. Thus, HO-1 induction is thought to be an adaptive response that offers cytoprotection to cells and tissues against oxidative stress [13].

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β (TGF-β) superfamily. BMPs were originally identified as cartilage and bone formation factors [14,15], but they also regulate cell growth, differentiation, chemotaxis, and apoptosis of various cell types during embryonic development and postnatal tissue remodeling. At the cell surface of target cells, BMP dimers bind type I (activin receptor-like kinase (ALK) 2, ALK3, or ALK6) and type II (BMPR-II, ActR-IIA, or ActR-IIB) BMP receptors, forming heteromeric complexes. The type II BMP receptor phosphorylates the type I BMP receptor, allowing it to phosphorylate the BMP receptor-regulated Smad (Smad1, Smad5, or Smad8) proteins. Upon activation, these Smads bind to Smad4 and translocate from the cytoplasm to the nucleus, where they regulate transcription of BMP target genes [16]. In addition to this Smad pathway, BMPs are known to activate mitogen-activated protein kinases (MAPKs) and cross-talk with other signaling pathways [17,18].

Multiple BMPs, BMP receptors, and intracellular signaling molecular are verified in the embryonic development and postnatal kidney [19]. Among them, BMP7 has received much attention as a renoprotective factor responsible for the maintenance of kidney homeostasis [20–22]. Although BMP6 and BMP7 are structurally similar and activate the same downstream signaling pathways [23], there are few reports concerning a possible role for BMP6 in kidney.

Abbreviations: BMP6, bone morphogenetic protein 6; HK-2, immortalized human kidney proximal tubule cells; HO-1, heme oxygenase 1; Act D, actinomycin D; CHX, cycloheximide; ROS, reactive oxygen species; HSP, heat-shock protein; MAPK, mitogen-activated protein kinase.

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Unlike BMP7, which is essential for early development and maintenance of the kidney, BMP6 is expressed in the kidney only toward late gestation [24,25]. Recently, it was found that downregulation of BMP6 in late gestation of intrauterine growth-restricted newborns may predispose to tubulointerstitial fibrosis in postnatal life. Tubulointerstitial fibrosis has been proposed as an antecedent of hypertension [26]. This suggests that BMP6 may have a role in repair and regeneration of kidney. However, it is unclear whether BMP6 has directly protective effects on renal cells. In this study, we evaluated the effects of BMP6 against oxidant injury induced by H_2O_2 in HK-2 cells. We also determined the molecular mechanisms involved and the possible relationship between HO-1 induction and the cytoprotective effects of BMP6.

Materials and methods

Cell culture

The immortalized human proximal tubule cell line HK-2 (Cell Culture Center, Beijing, China) was grown in DMEM/F12 medium containing 10% FBS and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin). Cells were maintained at 37 °C in a 100% humidified atmosphere of 5% CO_2 and 95% air. Fresh growth medium was added to the cells every 2 days until confluent. With the exception of the cells used for transfection, cells were growth-arrested in serum-free medium for 24 h before being used in experiments. All experiments were performed under serum-free conditions. Cell culture ingredients were obtained from Invitrogen–GIBCO (Grand Island, NY, USA). Recombinant human BMP6 protein was obtained from R&D Systems (Minneapolis, MN, USA).

Cell viability assay

Cell viability was determined using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) containing Dojindo's tetrazolium salt (WST-8). WST-8 was determined by its cellular dehydrogenase-dependent reduction to an orange formazan product that is soluble in cell culture medium. CCK-8 (10 μl/well) was added to the medium for 2 h at 37 °C. The absorbance was read at 450 nm with an enzyme-linked immunosorbent assay analyzer (Thermo Electron, Shanghai, China). Cell viability was also measured by using the trypan blue dye exclusion method. Both detached and attached cells were resuspended in cell culture medium, mixed with an equal volume of 0.4% (w/v) trypan blue dye (Sigma, St. Louis, MO, USA), and left at room temperature for 5 min. The proportion of cells remaining nonviable (unable to exclude trypan blue) was counted by using a hemocytometer and expressed as a percentage of the total number of cells.

Caspase-3 activity assay

Caspase-3 activity was assessed using a PE-conjugated monoclonal anti-active caspase-3 antibody (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, cells were collected by trypsinization followed by centrifugation. The cellular pellet was resuspended in Cytofix/Cytoperm buffer and incubated on ice for 20 min. Cells were resuspended in Perm/Wash buffer plus antibody and incubated for 30 min at room temperature. Fluorescence was measured on a FACScan flow cytometer (BD Biosciences).

RT-PCR assay

Total RNA was extracted using a TRIzol kit (Invitrogen, Carlsbad, CA, USA) and the concentration was detected using a spectrophotometer. The reverse transcription reaction was performed using 2 μg of total RNA, oligo$(dT)_{18}$ primer (Takara, Dalian, China), and MMLV reverse transcriptase (Promega, Madison, WI, USA). PCR was performed using the specific sense and antisense primers designed to recognize human sequences. The primers were as follows: HO-1 (NM_002133), 5′-TGAAGGAGGCCACCAAGGAG-3′ and 5′-GTGGGCCACCAGCAGCTC-3′; GAPDH (NM_002046), 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. PCR products were then separated electrophoretically in a 1.2% agarose DNA gel and stained with ethidium bromide for analysis with a UVItec Bioimaging System (UVItec, Cambridge, UK). The PCR cycle number for each primer pair was optimized for linear increases in densitometric band intensity measurements with increasing PCR cycles. Semiquantitative analysis of mRNA expression was accomplished by obtaining the ratio of the band density of the mRNA of interest to that of GAPDH (a housekeeping gene) from the same sample.

Western blot analyses

Protein expression and activation (via phosphorylation) of MAPKs were assayed by Western blot. The primary antibody for human HO-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used at 1:500 dilution, and those for ERK, pERK, p38, pp38, JNK, and pJNK were purchased from BD Bioscience and used at 1:2500 dilution. The secondary antibody (goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase) was purchased from Promega and used at 1:2500 dilution. In brief, cells were scraped with lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) plus protease inhibitor (2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM PMSF; Amresco, Solon, OH, USA). Lysates were clarified by centrifugation. The protein concentration in the supernatant was determined using the BCA assay (Pierce, Rockford, IL, USA) and 30 μg of protein was processed for SDS–PAGE, which was performed on 12% gels. The proteins were electrophoretically transferred to nitrocellulose membrane (Millipore, Bedford, MA, USA). The blots were blocked with 3% nonfat milk in Tris-buffered saline–0.05% Tween 20 (TBST; pH 8.0) and then incubated with primary antibodies, washed with TBST, and incubated with secondary antibodies conjugated with horseradish peroxidase. The protein bands were visualized with ECL reagents and subsequent exposure to autoradiography film (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The intensities of the immunoblots were quantified with a scanner coupled to a personal computer with UVIband software (UVItec).

HO activity assay

HO activity was measured by determining the level of bilirubin generated in isolated microsomes [27]. Cells were harvested and homogenized in a phosphate buffer (pH 7.4) and centrifuged at 1000 *g* for 10 min at 4 °C. The resulting supernatants were centrifuged at 100,000 *g* for 60 min at 4 °C. The pellet was suspended in potassium phosphate buffer (pH 7.4), followed by sonication on ice for 2 s, and the amount of protein was determined with the BCA protein assay. The reaction mixtures (200 μl), which contained 0.2 mM substrate hemin, 500 μg/ml cell lysate, 0.5 mg/ml rat liver cytosol as a source of biliverdin reductase, 0.2 mM $MgCl_2$, 2 mM glucose 6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, 1 mM NADPH, and 50 mM potassium phosphate buffer (pH 7.4), were incubated at 37 °C for 2 h in the dark. The reaction was terminated with 0.6 ml of chloroform. After extraction, the absorbance of the chloroform layer was measured spectrophotometrically. Bilirubin formation was calculated from the difference in absorption at 464 and 530 nm. HO activity was expressed as nanomoles of bilirubin formed per hour per milligram of protein.

Plasmid constructs and transfection assay

A constitutive expression vector, pCDNA-HO-1, carries the coding sequence of the human HO-1 gene subcloned into pCDNA 6A