Upregulation of Id-1 via BMP-2 receptors induces reactive oxygen species in podocytes

Gregor Pache,1 Christina Schäfer,2 Sebastian Wiesemann,1 Erik Springer,2 Max Liebau,1 Hans Christian Reinhardt,2 Christian August,3 Hermann Pavenstädt,2,∗ and Martin Johannes Bek2,∗

1Department of Medicine, Division of Nephrology and General Medicine, University Clinic of Freiburg, Freiburg; and 2Department of Medicine, Division of Nephrology and General Medicine, and 3Department of Pathology, University Clinic of Münster, Münster, Germany

Submitted 8 June 2004; accepted in final form 29 March 2006

Bone morphogenetic proteins (BMPs) are secreted signaling molecules, which play a major role in kidney development and disease. Here, we show the existence of mRNA for BMP-2 and for the BMP receptors BMPRIA, BMPRIB, BMPRII, ACVR1A, ACVR2, and ACVR2B in differentiated mouse podocytes and the protein expression of BMPRIA in human glomerular podocytes. BMP-2 dose dependently increases the free cytosolic Ca2+ concentration in podocytes proving the existence of a functional receptor in these cells. Recent data indicate that in a myoblastic cell line and in a breast cancer cell line, BMP-2 increases the expression of Id-1, a negative regulator of basic helix-loop-helix transcription factors, but the role of BMP-2-stimulated Id-1 expression in the kidney has not been further characterized. Here, we show that BMP-2 increases the expression of Id-1 in differentiated podocytes. To investigate a role of Id-1 for podocyte function, overexpression of Id-1 was induced in differentiated mouse podocytes. Id-1-overexpressing podocytes show an increased NADPH-dependent production of reactive oxygen species (ROS). This effect can be evoked by BMP-2 and can be antagonized by anti-Id-1 antisense oligonucleotides. The data indicate that BMP-2 may, via an increased expression of Id-1 and an increased generation of ROS, contribute to important cellular functions in podocytes. ROS supposedly play a major role in cell adhesion, cell injury, ion transport, fibrogenesis, angiogenesis and are involved in the pathogenesis of membranous nephropathy.

bone morphogenetic protein-2; cytosolic free calcium concentration

THE FAMILY OF BONE MORPHOGENETIC (BMP) proteins belongs to the transforming growth factor-β superfamily and acts as signaling molecules. They were originally identified by their ability to induce ectopic bone formation (7, 52, 55). So far, more than 12 different members of the BMP superfamily have been identified but knowledge about the role of BMPs and BMP receptors in the kidney is limited. BMPs bind to specific heteromeric complexes of two related serine/threonine kinase receptors, type I and type II receptors. Three BMP type I receptors (BMPRIA/ALK-3, BMPRIB/ALK-6, BMPR2) and three BMP type II receptors (ACVR1, ACVR2, ACVR2B) have been characterized. Different BMPs bind with different affinity to these BMP receptor complexes. BMPs exert profound and specific effects on the organogenesis of mammals. BMP-2, -4, and -7 have direct or indirect roles in regulation of ureteric branching morphogenesis and branch formation (32, 34). In BMP-7-deficient mice, metanephric mesenchymal cells fail to differentiate, resulting in a virtual absence of glomeruli in the kidneys of newborn mice (30). In diabetic rats, BMP-7, a closely related protein with great structural similarities to BMP-2 (46), partially reverses diabetic-induced kidney hypertrophy, hyperfiltration, urine albumin excretion, and glomerular histology (53). This effect could be mediated by TGF-β expression because BMP-7 antagonizes the TGF-β-dependent fibrogenesis in mesangial cells (54). Less is known about the role of BMP-2 in the kidney. Within the glomerulus BMP-2 inhibits mesangial cell proliferation induced by epidermal growth factor and platelet-derived growth factor (9). In addition, BMP-2 expression is increased in cultured mesangial cells exposed to elevated glucose concentrations, suggesting that BMP-2 plays a role in mesangial cell injury during diabetic nephropathy (33). Knowledge of the expression and function of BMPs and their respective receptors in podocytes, which are the target cell of injury in most proteinuric diseases, is limited.

Id gene products were first identified in myoblasts, where they prevent myogenic basic helix-loop-helix transcription factors (bHLH) from binding to muscle-specific regulatory elements (23, 38, 44). Id proteins dimerize with bHLH proteins to form heterodimers which are unable to bind DNA because Id proteins lack the basic domains for DNA interaction. A BMP-responsive element has been identified in the structure of Id-1 that has been implicated in the inhibition of myogenesis (19). Recently, it was shown that BMP-2 increases the expression of Id-1 in a myoblastic cell line and in a breast cancer cell line, but knowledge about the functional role of Id-1 expression in the kidney is limited. Several reports indicate that Id genes are downstream targets of TGF-β signaling (17, 20, 29, 39), which is a critical mediator of glomerulosclerosis (42). Because reactive oxygen species (ROS) can increase TGF-β activity and protein expression (25), we tested the hypothesis of whether BMP-2 stimulation via Id-1 modifies ROS generation in podocytes.

MATERIALS AND METHODS

Culture of podocytes. Differentiated immortalized mouse podocytes derived from mice that harbor a thermosensitive variant of the
SV40 large T-antigen inserted into the mouse genome were used (35). These mouse podocytes proliferate at 33°C in the presence of interferon-γ and differentiate at 37°C after removal of interferon-γ. Podocytes were maintained in RPMI 1640 medium (Life Technologies, Eggenstein, Germany) supplemented with 5% fetal calf serum (Biochrom, Berlin, Germany), 100 kU/l penicillin, and 100 mg/l streptomycin (Life Technologies). To propagate podocytes, cells were cultured at 33°C on type I collagen (permissive conditions) in culture medium supplemented with 10 U/ml recombinant interferon-γ (Roche, Mannheim, Germany). To induce differentiation, podocytes were maintained on type I collagen (Biochrom) at 37°C without supplementation with interferon-γ (nonpermissive conditions) for 10–14 days. Only differentiated podocytes between passages 12 and 17 were used in our experiments. Cells were switched to media that contained only 1% fetal calf serum 24 h before the experiments and then stimulated with BMP-2 (Research Diagnostics), vehicle, or various treatments. In Id-1-overexpressing podocytes, genitictin (150 µg/ml) was added to the medium to maintain selective conditions.

**RNA preparation.** Total cellular RNA from podocytes was isolated with guanidinium/acid phenol/chloroform extraction as described previously (5). The amount of RNA was measured photometrically. The integrity of RNA was analyzed after electrophoresis in a 1.5% agarose gel, ethidium bromide staining, and UV irradiation visualization.

**RT-PCR.** RT-PCR amplification was performed according to the methods described recently. Briefly, 0.2 µg of total RNA was mixed in 5× reverse transcription buffer containing 0.5 mM dNTP, 10 µM random primers, 10 mM dithiothreitol, 4 U ribonuclease inhibitor, and 20 U M-MLV reverse transcriptase (reverse transcriptase was also added to control for the amplification of contaminating DNA) for 60 min at 37°C. The cycle profile included denaturation for 60 s at 94°C, annealing for 1 min at temperatures given in Table 1, and extension for 60 s at 72°C. The primers used and the numbers of cycles performed for each primer pair are shown in Table 1. To analyze the amplification products, 10 µl from each PCR reaction were separated in a 1.5% agarose gel, followed by ethidium bromide staining and UV irradiation visualization.

**Measurements of intracellular free Ca2+ concentration.** Measurements of intracellular free Ca2+ concentration ([Ca2+]i) were performed by 10.220.32.247 on June 23, 2017 http://ajprenal.physiology.org/ Downloaded from

### Table 1. Primer sequences and amplification conditions

<table>
<thead>
<tr>
<th>Gene [Mus musculus] Abbreviation</th>
<th>Primers 5′−→3′ Forward/Reverse</th>
<th>Accession</th>
<th>bp</th>
<th>AT, °C</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2 (BMP-2)</td>
<td>AGT GGT TTC TGG TCG AAG TGC</td>
<td>NM_007553</td>
<td>345</td>
<td>62</td>
<td>32</td>
</tr>
<tr>
<td>BMP receptor type 1A (BMPR1a)</td>
<td>TAA AGG CCG CTA TGA AGA AGG</td>
<td>NM_009758</td>
<td>422</td>
<td>60</td>
<td>32</td>
</tr>
<tr>
<td>BMP receptor type 1B (BMPR1b)</td>
<td>CAC TCC CAT TGG TCA TGA AA</td>
<td>NM_007560</td>
<td>386</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td>BMP receptor type II (BMPR2)</td>
<td>TTC CAA TAT GCT TCA GCA TC</td>
<td>NM_007561</td>
<td>420</td>
<td>60</td>
<td>28</td>
</tr>
<tr>
<td>Activin A receptor type 1 (ACVR1)</td>
<td>GAT CAT TCA TGG AAG CAG</td>
<td>NM_007394</td>
<td>327</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Activin receptor II A (ACVR2)</td>
<td>AGG TCT CAC GGT CTT CAT TC</td>
<td>NM_007396</td>
<td>365</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Activin receptor II B (ACVR2b)</td>
<td>CTT TAA GCC CTT GCC TTT GC</td>
<td>NM_007397</td>
<td>355</td>
<td>60</td>
<td>38</td>
</tr>
</tbody>
</table>

AT, annealing temperature; BMP, bone morphogenetic protein.
was expressed as nanomoles O$_2$.

id-1 antisense oligonucleotide, 5'-H11032-GTACTTCCAGCGGTCACCGTCACGG-3';

CCACTGGCGACCTTCATG-3';

Measurement of NADPH-oxidase activity. Measurement of superoxide anion (O$_2^-$) production was performed as described recently (1).

Podocytes were rinsed once with cold PBS collected in Krebs solution (99 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl$_2$, 1.2 mM MgCl$_2$, 25 mM NaHCO$_3$, 1.03 mM K$_2$HPO$_4$, 20 mM Na-HEPES, 11.1 mM glucose, pH 7.35) and centrifuged (200 g, 4°C, 5 min). The supernatant was discarded and the cells were resuspended in fresh Krebs solution containing 5 μM lucigenin and stimulated with 100 μM NADPH. Bioluminescence was measured with Lumat LB9501 (Berthold, Wildbad, Germany). To calculate the amount of O$_2^-$, total counts were analyzed by calculating the areas under the curves (integration). O$_2^-$ generation was expressed as nanomoles O$_2^-$ generated per milligram of cellular protein per minute as described earlier (2). Protein content of the cell suspension was measured with the Lowry method.

Antisense experiments. Antisense experiments were performed using morpholino antisense oligonucleotides. The following oligonucleotides were used: id-1 antisense oligonucleotide, 5'-GGCACCTGGCCACCTTCATG-3'; control oligonucleotide (inverse antisense sequence), 5'-GTACTTCCAGGGTCACCGG-3'; (Gene Tools, Philomath, OR). Mouse podocytes were grown in six-well plates to 80% confluency. Antisense/control oligonucleotide (300 nmol) and ethoxylated-polyethylenimine (200 μM) were mixed in 600 μl reaction volume and incubated for 20 min at room temperature. After addition of 5.4 ml of serum-free culture medium to the oligonucleotide/ethoxylated-polyethyleneimine mixture, cells were incubated for 3 h with the oligonucleotide complex. The oligonucleotide complex was replaced by fresh serum-containing medium, and the cells were incubated for 16 h under standard incubator conditions. After stimulation with BMP-2 (10 μM) or vehicle for 1 h, cells were harvested for NADPH-oxidase activity measurement and Western blotting.

Immunohistochemical analysis. Fixation and preparation of tissue for immunohistochemical analyses were performed as described before (2). To summarize, human kidney tissues from patients after carci nonectomy were incubated in cold (4°C) PBS followed by 4% paraformaldehyde solution for 24 h at 4°C. Thereafter, kidney samples were embedded in paraffin and cut into 2-μm-thick slices. Tissue samples were deparaffinized in xylol for 1 h, gradually hydrated through graded alcohols (100 to 70%), and washed in deionized water. Antigen unmasking was performed by boiling the slices in citrate buffer for 10 min (10 mM sodium citrate). Blocking was performed using 1% BSA in PBS for 10 min. Sections were incubated for 24 h in a humidified chamber at 4°C with antibodies against BMP receptors (rabbit-anti-BMPR1A Abgent, AP2004b; goat-anti-BMPR2, R&D Systems, AF505; rabbit-anti-WT1, Santa Cruz, sc-192, 1:50 dilution). Following the application of a bridging antibody (DAKO, mouse antirabbit/goat, 1:125) and a second bridging antibody against mouse (DAKO 1:30), the immunoreactivity was determined by the APAAP-complex (1:100) using the Neufuchsin chromogene. Sections were examined by an experienced renal pathologist with a conventional light microscope (Zeiss LSM 510). Negative controls were performed by elimination or heat denaturation of the primary antibody. All procedures performed were in accordance with the ethic commission guidelines of The University of Muenster Ethic Commission.

Fig. 1. Ethidium bromide-stained agarose gel electrophoresis of RT-PCR products for bone morphogenetic protein (BMP)-2 and for the BMP receptors BMPR1A, BMPR1B, BMPR2, ACVR1A, ACVR2, and ACVR2B in differentiated mouse podocytes. Studies were performed with primers derived from published mouse cDNA sequences (Table 1). Experiments were performed by using RT (RT+) or no RT (RT−) in each set-up (RT− not shown). Amplification of GAPDH was used to prove RNA integrity. Sequence analysis of the resulting amplification products confirmed identity of the amplified fragments.

Fig. 2. A: effect of BMP-2 (10–8 M) on intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]) in podocytes grown with 1% fetal calf serum for 24 h. B: concentration-response curve of the effect of BMP-2 on [Ca$^{2+}$] in podocytes grown with 1% FCS for 24 h (n = 9–21, values are means ± SE, *P < 0.05 vs. 10–11 M, ANOVA, Scheffe’s test).
Immunocytochemistry. Fixation of differentiated podocytes grown on collagen-coated glass slides was performed by incubation with ice-cold methanol for 5 min followed by permeabilization with 0.3% Triton X-100 for 10 min at room temperature. Blocking was performed using a 1–2% BSA/0.2% fish gelatin solution for 30 min at room temperature. Thereafter, slides were incubated for 24 h in a humidified chamber at 4°C with antibodies against Id-1 (rabbit-anti-Id-1, Santa Cruz, sc-427, 1:200 dilution) followed by incubation with a FITC-labeled secondary antibody (donkey antirabbit, Jackson Immunoresearch).

Statistical analysis. Data expressed as means ± SE were analyzed by ANOVA for repeated measures for comparisons within groups and one-way ANOVA for comparisons among groups. Student’s t-test was used for a two-group comparison. \( P < 0.05 \) was considered statistically significant.

RESULTS

Expression of BMP-2 and BMP receptors in mouse podocytes. Figure 1 shows an ethidium bromide-stained agarose gel with PCR products for BMP-2 and for the BMP receptors BMPR1A, BMPR1B, BMPR2, ACVR1A, ACVR2, and ACVR2B in differentiated mouse podocytes. Studies were performed with primers derived from published mouse cDNA sequences (Table 1). Sequence analysis of the resulting amplification products revealed identity with published sequences.

BMP-2 increases \( [\text{Ca}^{2+}]_i \) in mouse podocytes. To evaluate the presence of functionally active BMP receptors in podo-

![Fig. 3. NADPH-dependent activation of superoxide anion (\( \text{O}_2^- \)) production is increased in BMP-2 (10 \( \mu \text{mol/l} \))-stimulated podocytes. A: time-dependent NADPH-mediated \( \text{O}_2^- \) production in podocytes and controls prestimulated with BMP-2 for 1 h. B: summary of the relative change in \( \text{O}_2^- \) production in podocytes stimulated with BMP-2 for 1, 4, and 24 h compared with vehicle (control)-treated cells. There is a significant increase after 1 h \(( n = 6/5, \text{values are means} \pm \text{SE, } *P < 0.05 \text{ controls, } t\text{-test})\).](http://ajprenal.physiology.org/)

![Fig. 4. A: Western blot showing the time-dependent upregulation of Id-1 after stimulation with BMP-2 for the indicated times. B: normalized summary of 4–5 experiments. Id-1 expression was significantly elevated after 0.5 and 4 h of stimulation. Ponceau staining confirmed equal amounts of total protein for all lanes (values are means \pm \text{SE, } *P < 0.05 \text{ vs. control, } t\text{-test}). C: Id-1 in untransfected differentiated podocytes localizes to the nucleus (left). Control experiments with denatured primary antibody reveal no staining (right).](http://ajprenal.physiology.org/)
cytes, differentiated podocytes were stimulated with BMP-2. At $10^{-8}$ M, BMP-2 induced a reversible biphasic increase in $[\text{Ca}^{2+}]$ (Fig. 2A). Reduction of the extracellular $\text{Ca}^{2+}$ from $2 \times 10^{-3}$ to $10^{-6}$ M did not change the BMP-induced peak but significantly diminished the plateau (data not shown), indicating that both $\text{Ca}^{2+}$ release from intracellular $\text{Ca}^{2+}$ stores (peak) and a transmembranous $\text{Ca}^{2+}$ influx (plateau) are responsible for the BMP-induced $[\text{Ca}^{2+}]_i$ increase. The effect of BMP on $[\text{Ca}^{2+}]_i$ was concentration dependent with a half-maximal concentration of $\sim 1 \text{nM}$ (BMP vs. $[\text{Ca}^{2+}]_i$ increase: $10^{-11}$ M = $29 \pm 17 \text{ nmol/l}$, $n = 10$; $10^{-10}$ M = $190 \pm 57 \text{ nmol/l}$, $n = 13$; $10^{-9}$ M = $273 \pm 81 \text{ nmol/l}$, $n = 19$; $10^{-8}$ M = $422 \pm 68 \text{ nmol/l}$, $n = 17$; $10^{-7}$ M = $372 \pm 69 \text{ nmol/l}$, $n = 21$; Fig. 2B).

BMP-2 increases NADPH-dependent generation of $\text{O}_2^-$ in mouse podocytes. Activation of the NADPH oxidoreductase enzyme complex leading to generation of ROS has been shown in podocytes in vivo and in vitro (2, 11, 12, 36). It has been suggested that this generation of ROS plays a major role in the pathogenesis of proteinuria (36, 47). BMP-7 has been shown to reverse proteinuria in diabetic rats to normal in a dose-dependent manner (53), but no information about the function of BMP-2 on proteinuria is available. However, opposing effects of BMP-2 and BMP-7 have been described (10, 48, 49). To evaluate whether BMP-2 is involved in the generation of ROS, we stimulated mouse podocytes with either vehicle or BMP-2 (10 μmol/l) for 1, 4, and 24 h and measured NADPH-oxidase activity thereafter. The dosage of 10 μmol/l was choosen because 10 μmol/l BMP-2 induced a strong expression of Id-1 in differentiated podocytes (see Fig. 4). The total amount of superoxide anions generated during the experimental period (15 min) was calculated by integration, and control cells were compared with BMP-2-stimulated cells. Figure 3A shows that the addition of NADPH (0.1 mM) to control podocytes led to an increase in $\text{O}_2^-$ production after 1 h of prestimulation with BMP-2. Following addition of NADPH (0.1 mM) to BMP-2-treated podocytes, the generation of $\text{O}_2^-$ production was increased by $\sim 1.8$-fold after 1 h. Figure 3B shows the time dependency of NADPH production in podocytes treated with either vehicle or BMP-2. Significant upregulation of NADPH production is seen only at 1 h.

BMP-2 induces the expression of Id-1 in podocytes. To obtain more information about BMP-2-induced functions in podocytes, we determined Id-1 expression in podocytes stimulated with BMP-2 (10 μmol/l) or vehicle. Recent data indicate that BMP-2 increases Id-1 expression in a myoblastic cell line and in a breast cancer cell line, but the role of BMP-2 stimulation on Id-1 expression in podocytes has not been further characterized (6, 20). Western blot analysis of mouse podocytes stimulated with BMP-2 (10 μmol/l) or vehicle indicates that Id-1 protein expression is significantly upregulated in BMP-2-stimulated podocytes compared with controls. An increase in Id-1 expression (Id-1 $\sim 18$ kDa) can be seen at 0.5, 4, and 24 h (Fig. 4A), but significance was reached only at 0.5 and 4 h (Fig. 4B). A doublet band was detected for Id-1, in both untransfected and Id-1-overexpressing cells. This doublet band most likely represents a glycosylated form of Id-1. The protein sequence contains a putative O-glycosylation site at a threonine in position 75, as well as putative phosphorylation

![Fig. 5. NADPH-dependent activation of superoxide anion production is increased in Id-1-overexpressing podocytes. A: Western blot performed with antibodies against Id-1 showing the expression levels of Id-1 in overexpressing podocytes compared with podocytes transfected with vector only. B: summary of 3 experiments (values are means ± SE, *$P < 0.05$ vs. control, t-test). C: time-dependent NADPH-mediated superoxide anion production in Id-1-overexpressing podocytes and controls. D: summary (n = 6) of calculated integrals in Id-1-overexpressing podocytes and controls after stimulation with NADPH as a substrate. To calculate the amount of superoxide produced, total counts were generated by integration of the signals (values are means ± SE, *$P < 0.05$ vs. control, t-test).](http://ajprenal.physiology.org/)

sites for protein kinase A, protein kinase C, protein kinase CKI, and protein kinase GSK3, which all might explain the doublet band. To further test expression and localization of Id-1 in differentiated podocytes, immunocytochemistry in unstimulated podocytes was performed. As expected, Id-1 was found exclusively in the nucleus.

**Id-1-overexpressing podocytes show an increased NADPH-dependent generation of $O_2^-$ in podocytes.** To demonstrate that the effects of BMP-2 on cellular ROS generation could be replicated by Id-1, a mouse podocyte cell line overexpressing Id-1 was created. Expression of Id-1 was significantly (~18-fold) increased in Id-1-transfected cells compared with vector only-transfected cells (controls; Fig. 5A). The results of three Western blot experiments are summarized in Fig. 5B. These cell lines were used to measure NADPH-dependent $O_2^-$ production. Addition of NADPH (0.1 mM) to Id-1-overexpressing podocytes significantly increased $O_2^-$ production (5-fold) compared with vector only-transfected cells (controls; Fig. 5C). The total amount of $O_2^-$ generated during the experimental period (15 min) was again calculated by integration and vector only-transfected cells were compared with Id-1-overexpressing cells. Figure 5D summarizes these experiments.

**Anti-Id-1 antisense oligonucleotides inhibit BMP-2-induced NADPH-dependent generation of $O_2^-$ in podocytes.** To prove that BMP-2 is responsible for the generation of $O_2^-$ via the Id-1 pathway, experiments with Id-1-antisense oligonucleotides were performed. Stimulation of untransfected mouse podocytes with vehicle or BMP-2 (10 μmol/l) for 1 h again showed a significant increase in the generation of $O_2^-$ production in BMP-2-treated cells compared with vehicle-stimulated control cells (Fig. 6). Pretreatment of BMP-2-stimulated (10 μmol/l) podocytes with Id-1-antisense oligonucleotides significantly inhibited the amount of superoxide anions generated by the addition of NADPH and reduced protein expression for Id-1 compared with inverse antisense (control oligonucleotides)-treated cells. The amount of $O_2^-$ produced in antisense-treated podocytes was not significantly different from vehicle-stimulated control cells (Fig. 6). In contrast, pretreatment of BMP-2-stimulated (10 μmol/l) podocytes with control oligonucleotides had no inhibitory effect on NADPH-induced ROS production and on Id-1 protein expression.

**Expression of BMP receptors in the glomerulus.** To investigate the protein expression of BMP receptors in the glomerulus, immunohistochemical staining was performed with antibodies recognizing all known BMP receptors. Unfortunately, all commercially available antibodies tested were unsuitable for immunohistochemical staining in mouse and rat tissues and most antibodies tested were unsuitable in human tissues despite specific declarations on specification sheets. Figure 7 shows expression of the BMPR1A receptor in human podocytes. In contrast to our PCR data, the BMPR2 receptor was not found in the glomerulus or elsewhere in the kidney but could be clearly distinguished on monocytes found in the glomerulus (Fig. 7).

**DISCUSSION**

BMPs regulate important cellular functions such as differentiation of pluripotent mesenchymal cells into the osteogenic lineage and the function of differentiated osteoblasts (30, 52, 55, 56). Within the kidney, BMP-2 is expressed by metanephric mesenchymal cells and inhibits collecting duct morphogen-
BMP-2 exerts its effect via type I and type II transmembrane serine threonine kinase receptors to form heteromeric receptor complexes with subsequent phosphorylation of type I receptors and activation of the catalytic activity of type I receptor kinase (21). The simultaneous expression of BMP-2 and BMP type I and type II receptor mRNA in podocytes suggests a modulation of podocyte function by autocrine or paracrine regulation. Recent data indicate that BMP-2 is present in mesangial cells of the glomerulus, too (33).

In the present study, we show that BMP-2 increases \([\text{Ca}^{2+}]_i\) in podocytes concentration dependently, suggesting the presence of functionally active BMP receptors and a regulatory role of BMP-2 for \(\text{Ca}^{2+}\)-dependent signaling pathways in podocytes. To our knowledge, a BMP-induced calcium increase has not been shown so far. BMP-2 significantly increased \([\text{Ca}^{2+}]_i\) at concentrations between \(10^{-10}\) and \(10^{-7}\) M, suggesting an interaction with a high-affinity type I receptor such as BMPR1A, BMPR1B, or ACVR1. This fits nicely with the expression of the BMPR1A receptor in podocytes in vivo. BMP-2 induced a biphasic \([\text{Ca}^{2+}]_i\), response consisting of an initial \(\text{Ca}^{2+}\) peak followed by a sustained \(\text{Ca}^{2+}\) plateau, the latter being dependent on extracellular \(\text{Ca}^{2+}\) influx. Stimulation of calcium-dependent PKC activity by BMP-2 has been shown (4, 16), but the PKC isoform involved has not been further characterized. Recently, we demonstrated that \([\text{Ca}^{2+}]_i\)-mobilizing agonists induce NADPH-oxidase activity in podocytes, a major source for the production of \(\text{O}_2^-\) in these cells (12).

In this study, we can show that BMP-2 increases NADPH-dependent generation of \(\text{O}_2^-\) in podocytes, an effect that is mediated by Id-1, a negative regulator of bHLH transcription factors, because pretreatment with Id-1 antisense oligonucleotides abolished this effect. In addition, \(\text{O}_2^-\) production was strongly increased in Id-1-overexpressing podocytes. Recently,
it was shown that transcription of Id-1 can be mediated by the early response gene Egr-1 (50). These data fit well to our finding in EGR-1-overexpressing human proximal tubule cells where an increase in \( \text{O}_2 \) production similar to that found in BMP-stimulated and Id-1-overexpressing podocytes was found (1). Recent data show that Id-1 is crucial for the formation of intact neovascularature (31, 44). In addition, elevated levels of Id-1 have been found in synovial neovascularization of patients with rheumatoid arthritis (45). ROS might mediate this effect because NAD(P)H oxidase-dependent vascular endothelial growth factor-induced signaling and angiogenesis have been shown (51).

Interestingly, Id genes are downstream targets of TGF-\( \beta \) signaling (17, 20, 29). In the kidney, TGF-\( \beta \) plays an important role in the pathogenesis of glomerulosclerosis, the end stage of many glomerulopathies (18, 42, 54). The BMP-2-induced up-regulation of Id-1 protein expression and NADPH-oxidase activity could contribute to glomerulosclerosis, because ROS can increase TGF-\( \beta \) activity and protein expression in the kidney (18, 22, 25, 42). In our experiments, an increased ROS generation following BMP-2 stimulation was present only after 1 h of stimulation while Id-1 protein expression was still markedly increased after 4 h of stimulation. This effect could be caused by the interaction of Id-1 with other transcription factors that alter gene regulation or by signal transduction pathways downstream of Id-1 with deactivation of the NADH/ NADPH-oxidase complex. Several signal transduction components essential for the actions of BMP-2 downstream of the serine/threonine kinase receptor have been demonstrated, including SMAD proteins (28), mitogen-activated protein kinase (15), TGF-activated kinase 1 (24, 40), MAPK/ERK kinase (8), and protein kinase C (16). Recent data indicate that the mode of BMP receptor oligomerization seems to determine different BMP-2 signaling pathways (37).

Our data might give additional insight into podocyte function, because ROS seem to play an important role as intra- and extracellular messenger in renal disease. For instance, high glucose-induced activation of PKC supposedly plays an important role in ROS generation and renal injury in diabetic nephropathy. In addition, in Heymann nephritis C5b-9 attack on podocytes causes upregulation and translocation of the NADPH oxidoreductase enzyme complex to the cell membrane. Subsequently, ROS induce lipid peroxidation and degradation of glomerular basal membrane collagen IV, leading to proteinuria (22, 36, 47). BMP-2 may therefore contribute to the pathogenesis of proteinuria via activation of NADPH oxidases. An induction of granulocyte macrophage colony-stimulating factor has been found in podocytes stimulated with ROS (11). However, other roles for BMP-2 in podocytes seem possible, too. A BMP-responsive element has been identified in the structure of Id-1 that has been implicated in the inhibition of myogenesis (19). In addition, a direct suppression of the myogenic phenotype has been shown for BMP-2 (27). This effect might be regulated by ROS, because inhibition of myogenesis through redox-dependent mechanisms has been shown (26, 41). Hence, it seems likely that BMP-2-induced generation of ROS alters gene expression and inhibits cellular functions in podocytes.

In summary, we show in this study that cultured podocytes express mRNA for BMP-2 and BMP receptors. Activation of these receptors by BMP-2 can be demonstrated by an increase of \([ \text{Ca}^{2+} \]) and via upregulation of Id-1 to subsequent generation of \( \text{O}_2 \). Further studies will be necessary to determine the mechanisms involved in Id-1-dependent ROS generation.

**ACKNOWLEDGMENTS**

We thank C. Hupfer, P. Daemisch, P. Kulick, and M. Wolters for excellent technical assistance. Podocyte cells were a generous gift from P. Mundel (Albert Einstein College of Medicine, New York, NY).

**GRANTS**

This study was supported by Deutsche Forschungsgemeinschaft PA 483/5–1.

**REFERENCES**


16. Hay E, Lemonnier J, Fromigne O, and Marie PJ. Bone morphogenetic protein-2 promotes osteoblast apoptosis through a Smad-independent,


