Morphine stimulates platelet-derived growth factor receptor-β signalling in mesangial cells in vitro and transgenic sickle mouse kidney in vivo

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Pain and end-stage renal disease are associated with reduced survival and poor quality of life in sickle cell disease (SCD).1–4 Morphine and its congeners are used to treat severe pain after vasoocclusive crises and ongoing chronic pain in SCD.5,6 As a possible complication of this therapeutic approach, we observed previously that clinically relevant doses of morphine incite kidney pathology, glomerular enlargement, and albuminuria in wild-type and transgenic sickle mice.7–9 Here, we investigate the mechanism(s) by which morphine stimulates renal disease.

In addition to their induction of analgesia, opioids activate growth, survival, and cytoprotection via opioid receptors (OPs) on multiple cell types including glomerular mesangial cells.7,10–15 Mitogenic signalling induced by morphine involves co-activation of receptor tyrosine kinases (RTKs) for vascular endothelial growth factor receptor-2 and platelet-derived growth factor receptor-β (PDGF-β) and downstream mitogenic signalling of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and signal transducer and activator of transcription 3 (Stat3) pathways via µ-opioid receptor (MOP) on endothelium and pericytes.16–19 PDGF-β signalling is critical to mesangial function, because overexpression or deletion of PDGF-B or PDGF-β influences mesangial proliferation and glomerular pathology in the kidney.20

We investigated the mitogenic signalling of morphine in vitro in the mesangial cells and in vivo in the kidneys of transgenic mice expressing sickle haemoglobin (HbS) or normal human haemoglobin (HbA). Furthermore, we examined whether treatment with morphine influenced renal function in HbS mice.

**Editor’s key points**

- Morphine can worsen kidney disease in sickle cell disease (SCD).
- This study investigated the effects of morphine on mesangial cells in vitro and mouse kidneys in vivo.
- Morphine activated several key mitogenic signalling pathways both in vitro and in vivo.
- Morphine worsened renal function in sickle cell mice.
- Inhibition of the identified mitogenic pathways may prevent morphine-induced renal damage in patients with SCD.

**Background.** Pain and renal dysfunction occur in sickle cell disease. Morphine used to treat pain also co-activates platelet-derived growth factor receptor-β (PDGFR-β), which can adversely affect renal disease. We examined the influence of morphine in mesangial cells in vitro and in mouse kidneys in vivo.

**Methods.** Mouse mesangial cells treated with 1 µM morphine in vitro or kidneys of transgenic homozygous or hemizygous sickle or control mice (n = 3 for each), treated with morphine (0.75, 1.4, 2.14, 2.8, 3.6, and 4.3 mg kg⁻¹ day⁻¹ in two divided doses during the first, second, third, fourth, fifth, and sixth weeks, respectively), were used. Western blotting, bromylated deoxy uridine incorporation-based cell proliferation assay, reverse transcriptase-polymerase chain reaction, immunofluorescent microscopy, and blood/urine chemistry were used to analyse signalling, cell proliferation, opioid receptor (OP) expression, and renal function.

**Results.** Morphine stimulated phosphorylation of PDGFR-β and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) to the same extent as induced by platelet-derived growth factor-BB (PDGF-BB) and promoted a two-fold increase in mesangial cell proliferation. The PDGFR-β inhibitor, AG1296, OP antagonists, and silencing of µ- and κ-OP abrogated morphine-induced MAPK/ERK phosphorylation and proliferation by ≏100%. Morphine treatment of transgenic mice resulted in phosphorylation of PDGFR-β, MAPK/ERK, and signal transducer and activator of transcription 3 (Stat3) in the kidneys. Morphine inhibited micturition and blood urea nitrogen (BUN) clearance and increased BUN and urinary protein in sickle mice.

**Conclusion.** Morphine stimulates mitogenic signalling leading to mesangial cell proliferation and promotes renal dysfunction in sickle mice.

**Keywords:** morphine; nephropathy; pain; platelet-derived growth factor; sickle cell disease

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Methods

All mouse studies were performed after receiving approval from our Institutional Animal Care and Use Committee (ID#0806A37663).

Mice

Homozygous (BERK), hemizygous (hBERK1), and control (HbA-BERK) transgenic mice were used. BERK, the homozygous sickle mice, have knockout of both murine α- and β-globins and carry the linked transgenes for human α- and β-globins and express >99% human HbS. BERK mice present the sickle phenotype of human disease including renal disease, haemolysis, reticulocytosis, anaemia, organ damage, pain, and reduced survival. hBERK1 mice are hemizygous for knock-out of murine β-globin, but are homozygous for knockout of murine α-globin and carry a single copy of the linked transgenes for human α- and β-globins. They express ~25% human HbS and show kidney pathology. All three types of mice are littermates and hence have similar genetic background. Mice were bred <12 h light-to-dark cycle, in our pathogen-free facility and phenotyped by isoelectric focusing for Hb and by real-time polymerase chain reaction for transgene number.

Cell culture

Mouse mesangial cells (American Type Culture Collection, Manassas, VA, USA) were cultured using a 3:1 mixture of Dulbecco’s modification of Eagle’s medium and F12-Hams medium, respectively, supplemented with 10% heat-inactivated fetal calf serum, 100 U ml⁻¹ penicillin, 100 U ml⁻¹ streptomycin, and 10 mM HEPES (all from Invitrogen, Carlsbad, CA, USA). Cells were stimulated with 1 μM morphine or 20 ng ml⁻¹ PDGF-BB (Invitrogen) in the presence or absence of the PDGFR tyrosine kinase inhibitor, AG-1296 (10 μM; Cayman Chemicals, Ann Arbor, MI, USA), naloxone (1 μM), methylnaltrexone (MNTX) 0.1 μM (Wyeth Pharmaceuticals, Philadelphia, PA, USA) and norbinaltorphimine (nor-BNI) 1 μM (Sigma-Aldrich, St Louis, MO, USA).

Drugs and treatments

Six each of HbA-BERK and hBERK1 and 12 BERK mice were divided into two equal groups and s.c. injected with either phosphate-buffered saline (PBS) or morphine sulphate (Baxter Esilderle Healthcare, Cherry Hill, NJ, USA) daily (0.75, 1.4, 2.14, 2.8, 3.6, and 4.3 mg kg⁻¹ divided doses in Weeks 1–6, respectively; equivalent to ~50–301 mg in a 70 kg human per day). At the end-point, mice were killed with compressed carbon dioxide and the kidneys were removed immediately for analysis.

MOP-1 and KOP-1 gene silencing

A cocktail of sequences of siRNA specifically targeting the mouse MOP-1 and KOP-1 genes (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used (for further information see Supplementary material).

Each siRNA consisted of a pool of three-target-specific 20–25 nucleotide siRNAs. On Day 1, mouse mesangial cells were plated in a six-well plate. On Day 2, 100 nM siRNA and 4 μl of transfection agent si-PORT Lipid (Ambion, Inc., Austin, TX, USA) were separately diluted with OPTI-MEM I medium (Invitrogen) and then mixed rapidly and incubated at room temperature for 15 min to form a complex. The siRNA/ transfection agent complex was overlaid on the mesangial cells drop wise. Control, scramble-siRNA (Santa Cruz Biotechnology, Inc.) was used as a negative control. After fourth incubation, an equal volume of mesangial cell growth medium was added to the cells followed by overnight incubation. Cells were then used to determine gene expression by reverse transcription–polymerase chain reaction (RT–PCR) or were used for other experiments.

Western immunoblotting

Kidney and mesangial cell lysates containing 100 μg of protein were resolved on a 3–15% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA, USA) as described previously. For immunoblotting, we used antibodies to phospho-p44/42 MAPK/ERK Thr 202/Tyr 204 (1:500), total p44/42 MAPK/ERK (1:500), phospho-Stat3 Tyr 705 (1:500), phospho-Stat3 Ser 727 (1:500), and total-Stat3 (1:500) from Cell Signalling Technology (Beverly, MA, USA); phospho-PDGFR-β Tyr 716 (1:250) from Upstate (Lake Placid, NY, USA); total PDGFR-β (1:500) from Cell Signalling Technology (Beverly); Thy-1(1:200). Immunoreactive proteins were seen using a species-specific antibody linked to alkaline phosphate followed by development of chemiluminescent signals using the ECF western blotting system (Amersham Life Sciences, Buckinghamshire, UK). Chemiluminescent signals were acquired using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and densitometric analysis was performed using Image J software (NIH, Bethesda, MD, USA).

Proliferation assay

Proliferation of mesangial cells was measured using bromylated deoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. Briefly, 2000 cells per well were seeded on a 96-well plate in 10% fetal calf serum containing mesangial cell medium. Cells were allowed to adhere to the plate overnight and then incubated overnight with 0.1% fetal calf serum containing medium. Cells were pre-incubated with AG-1296, naloxone, nor-BNI, MNTX, or sunitinib (2 μM, Pfizer, New York, NY, USA) for 60 min; then morphine or PDGF-BB was added for an additional 48 h. Appropriate control wells were used for each experiment.

Reverse transcription–polymerase chain reaction

Total RNA was isolated using Trizol reagent from the kidneys of 5 HbA-BERK, 7 hBERK1, and 6 BERK. Five micrograms of total
Results

Morphine stimulates mitogenic signalling by co-activating PDGFR-β via OPs in kidney mesangial cells

Mesangial cells stimulated with morphine in vitro showed a time-dependent increase in the phosphorylation of PDGFR-β and MAPK/ERK (Fig. 1A). Morphine-induced phosphorylation of MAPK/ERK was abrogated by naloxone and by AG1296, a pharmacological inhibitor of PDGFR, suggesting that morphine stimulates mitogenic signalling via OPs and PDGFR-β (Fig. 1A). PDGF-BB also stimulated MAPK/ERK phosphorylation, which was inhibited by AG1296 or naloxone, indicative of a cross-talk between PDGFR-β and OPs. Silencing of MOP and KOP on mesangial cells inhibited morphine-induced phosphorylation of MAPK/ERK, suggesting that morphine stimulates MAPK/ERK signalling via MOP and KOP (Fig. 1C). Interestingly, silencing of MOP and KOP also inhibited PDGF-BB-induced MAPK/ERK phosphorylation, indicating that OPs may contribute to PDGFR-β signalling (Fig. 1A). MAPK/ERK phosphorylation by morphine or PDGF-BB was inhibited by OP antagonists, MNTX and nor-BNI (Fig. 1C and D). These data show that morphine acts via OPs to stimulate mitogenic signalling by co-activating PDGFR-β.

Morphine-induced mesangial cell proliferation depends upon OPs, and PDGFR-β

Morphine-induced proliferation was prevented by PDGFR inhibition using AG1296 or sunitinib (Sutent), a clinically used inhibitor of PDGFR and other growth factor receptors (Fig. 2). Similarly, morphine-induced proliferation was abrogated by OP antagonism using naloxone, MNTX, or nor-BNI. Sutent and nor-BNI also inhibited the basal proliferation of mesangial cells, while naloxone and MNTX did not have a significant effect when compared with PBS.

MOP and KOP expression is increased in the sickle kidney

Kidneys of all three types of mice showed the expression of MOP and KOP, but not that of DOP (Fig. 3A and B). However, the expression of both MOP and KOP was significantly higher in BERK kidneys when compared with HbA-BERK. MOP and KOP expression was increased throughout the kidney in the BERK sickle mouse kidney including the glomerular epithelium and mesangium (Fig. 3C). In HbA-BERK, MOP was weakly expressed throughout the kidney, whereas KOP appeared to be strongly expressed on the vasculature when compared with other cells of the HbA-BERK kidney. More than one band for MOP was seen in HbA-BERK kidney when compared with a single band in sickle kidneys (Fig. 3A).

Morphine treatment of mice promotes mitogenic signalling in the kidney

Treatment of HbA-BERK, hBERK1, and BERK mice for 6 weeks with morphine resulted in increased phosphorylation of PDGFR-β, Stat3, and MAPK/ERK, when compared with PBS-treated
Fig 1  Morphine signalling in mouse mesangial cells in vitro. (A) Stimulation of mesangial cells with 1 μM morphine showing a time-dependent increase in phosphorylation of PDGFR-β and MAPK/ERK. (B) Morphine- and 20 ng ml⁻¹ PDGF-BB-induced PDGFR-β and MAPK/ERK phosphorylation are inhibited by 10 μM AG1296, a PDGFR-β inhibitor and naloxone, a non-selective OP antagonist. (C and D) Silencing of MOP and KOP on mesangial cells inhibits morphine- and PDGF-BB-induced MAPK/ERK phosphorylation. (E and F) OP antagonist, MNTX (0.1 μM) and nor-BNI (1 μM), inhibit morphine- and PDGF-BB-induced MAPK/ERK phosphorylation. Mean (SD), n=3.
mice (Fig. 4). Expression of the mesangial cell-specific protein, Thy1, also increased in morphine-treated kidneys. Kidneys of sickle BERK mice, which have the highest level of HbS expression, showed higher constitutive phosphorylation of PDGFR-β, Stat3, and MAPK/ERK and Thy1 expression when compared with HbA-BERK or hBERK1. Morphine further activated these signalling pathways in BERK.

Morphine treatment influences renal function in sickle mice

Morphine treatment of BERK mice for 6 weeks led to complete inhibition of micturition in three of six mice, while the other three mice showed 60% decreased urinary volume when compared with PBS-treated mice over a 24 h period (Fig. 5A). BUN levels increased while BUN clearance decreased significantly upon morphine treatment when compared with PBS (P<0.01; Fig. 5a and c). A ~2.5-fold increase occurred in urinary protein in morphine when compared with PBS-treated BERK mice (P<0.01, vs PBS) (Fig. 5a).

Discussion

We demonstrate that chronic exposure to morphine stimulates mitogenic signalling via co-activation of PDGFR-β in the kidney of control and sickle mice. Notably, morphine also impaired renal function in BERK sickle mice. Morphine stimulates mesangial cell proliferation through OP-mediated mitogenic signalling, which may in part contribute to renal disease. As morphine is often used as an analgesic for chronic pain, its use may exacerbate existing renal disease in SCD. We identify several molecular components critical to morphine’s mitogenic activity in the kidney, including OPs and PDGFR-β, which could potentially be targeted with FDA-approved drugs, such as MNTX and PDGFR-β inhibitors, to prevent morphine-induced renal injury without compromising analgesia.

Opioids and their receptors expressed in the central nervous system have also been demonstrated in the kidney. We observed an increase in expression of MOP and KOP in transgenic sickle mouse kidneys when compared with control mice. While it is unclear whether increased MOP and KOP expression is a cause or consequence of renal disease, our OP antagonism and silencing studies clearly show that morphine-induced renal effects are mediated by OPs. Previous studies suggest that morphine contributes to mesangial expansion by increasing glomerular mesangial cell proliferation, matrix synthesis, and immune complex formation. In the current study, using both in vitro mesangial cells and in vivo mouse models, we find that morphine stimulates mesangial cell proliferation, via phosphorylation of PDGFR-β and MAPK/ERK signalling, suggesting that morphine has a mesangial cell-specific effect. Notably, our finding that BERK kidneys express higher levels of MOP and KOP compared with PBS-treated BERK mice is consistent with the higher levels of constitutive phosphorylation of growth signalling pathways and Thy1 protein expression seen in BERK kidneys when compared with HbA-BERK kidneys. Thus, our study provides clear in vivo evidence that morphine promotes mitogenic signalling in the kidney.

PDGFR-β, Stat3, and MAPK/ERK signalling are key regulatory pathways in cellular proliferation and kidney disease. OPs activate MAPK/ERK and Stat3 phosphorylation and co-activate RTKs for growth factors including PDGFR-β. Deletion of PDGF-B or PDGFR-β results in the loss of mesangium in the glomerulus, while systemic overexpression of PDGF-B leads to mesangioproliferative alterations and renal fibrosis. Morphine stimulates PDGF-BB expression in endothelial cells. Therefore, it is likely that co-activation of PDGFR-β may amplify its mitogenic signalling in the kidney, which is consistent with our earlier observations on morphine-induced kidney growth, mesangial proliferation, and glomerular volume expansion in wild-type and sickle mice.

Glomerulomegaly may be a precursor to glomerular sclerosis and result in increased urinary protein excretion. Morphine-induced mesangial signalling may contribute to impairment in renal function in sickle mice. However, morphine also induces vacuolization of tubular epithelium and tubular damage in mice and rats. It is likely that morphine-induced tubular pathology in part contributes to proteinuria observed in BERK mice treated with morphine for 6 weeks. Our observations are consistent with the significantly increased BUN in Wistar rats treated with morphine for 30 days. Morphine also inhibited micturition in sickle mice similar to that observed clinically because of bladder retention caused by lowering of perfusion pressure and possibly by inducing renal pathology in these mice observed by us earlier. Therefore, chronic morphine treatment leads to renal dysfunction in sickle mice.

To prevent the inadvertent effect of morphine on renal disease, we identified OPs and PDGFR-β, as targets of its mitogenic activity. Earlier, we identified KOP as one of the targets of morphine activity in the kidney using OP-specific knockout
mice. In the present study, we used pharmacological and genetic approaches to identify the receptors associated with the activity of morphine in the mesangial cells. Pharmacologically, both nor-BNI and MNTX inhibited morphine-induced signalling and proliferation, suggestive of the OP-mediated effect. The pKi of nor-BNI for KOP was shown to be 0.1 nM, and for MOP of 102 nM. MNTX has a pKi of 8.0, 7.5, and 6.2 for MOP, KOP, and DOP, respectively. Therefore, based on pharmacological inhibition, it is likely that morphine acts via MOP, KOP, or both agonism on mesangial cells. This is similar to our observations showing inhibition of morphine signalling after silencing of either MOP or KOP. It is therefore likely that morphine acts via more than one OP.

Splice variants of MOP have been suggested to play a physiologically important role through heterodimerization. Multiple bands of MOP observed by us are suggestive of the presence of splice variants and perhaps heterodimerization of MOP with KOP. Although not known in the kidney, OPs are known to act as heterodimers in some cell types and therefore silencing or antagonizing one receptor may abrogate the activity of
Fig 4  Morphine signalling in kidneys of mice treated with morphine (MS) or PBS for 6 weeks. Density of phospho-protein bands was determined relative to respective total protein bands or with respect to glyceraldehyde-3-phosphate dehydrogenase. Each band is representative of three separate mouse kidneys. Mean (SD), n=3.

Fig 5  Renal function in BERK mice treated with morphine (MS) or PBS for 6 weeks. Three of six mice did not urinate in the 24 h period after 6 weeks of MS treatment, but all PBS-treated mice urinated. Therefore, n=6 for each data point except for MS in (c and d) where n=3. *P<0.01 vs PBS.
the other. An advantage of targeting KOP is that it may not antagonize morphine-analgesia, while MOP antagonists may block morphine-analgesia. It is possible that chronic treatment with morphine may lead to tolerance and therefore we administered escalating doses of morphine in our study. While activity of morphine via OPs may be reduced because of desensitization of OPs, it may act via non-opioid family of receptors. Indeed, morphine has been shown to bind to toll-like receptor 4 and promote neuroinflammation.12 Therefore, targeting RTKs with Sunitinib-like clinically used drugs may prevent the inadvertent effect of morphine in the kidney. However, Sunitinib significantly inhibited mesangial cell proliferation, supporting the notion that RTKs are critical to the maintenance of normal mesangium. Therefore, doses of RTK inhibitors must be carefully determined or it may be safer to use OP antagonists, such as MNTX, to block morphine’s mitogenic effect. The mechanistic understanding herein provides insights into ameliorating the inadvertent mitogenic activity of morphine in the kidney. As these studies are performed in mice, any alteration in clinical decision-making should not be made until proved in human studies.

Supplementary material
Supplementary material is available at British Journal of Anaesthesia online.

Authors’ contributions
M.L.W. performed morphine treatments and tissue processing, analysed data, and assisted with experimental design and writing; C.C. performed western immunoblotting and proliferation assay; Y.L. did western immunoblotting and RT–PCR; J.N. and T.P. performed immunofluorescence microscopy; and M.F. performed RT–PCR; R.P.H. genotyped and provided mice and advice on editing the manuscript; K.G. conceived, and M.L.W. performed morphine treatments and tissue processing, analysed data, and assisted with experimental design and writing; C.C. performed western immunoblotting and prolifer-

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Declaration of interest
None declared.

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