Morphine Induces Mesangial Cell Proliferation and Glomerulopathy Via Kappa Opioid Receptors

Marc L Weber, Mariya Farooqui, Julia Nguyen, Michael Ansonoff, John E Pintar, Robert P Hebbel and Kalpna Gupta

Division of Renal Diseases and Hypertension, and Division of Hematology, Oncology and Transplantation, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455, and Department of Neuroscience and Cell Biology, UMDNJ-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854.

Running headline: Morphine stimulates glomerular alterations

Address correspondence to: Kalpna Gupta, PhD
Mayo Mail Code 480
420 Delaware Street SE
Minneapolis, MN 55455
Telephone: 612-624-0123; fax: 612-625-6919
email: gupta014@umn.edu
**ABSTRACT**

Morphine sulfate (MS) stimulates mesangial cell (MC) proliferation, a process central to development of glomerular disease. The purpose of this study was to examine if specific opioid receptors (OR) and signal transducer and activators of transcription 3 (STAT3) signaling are associated with MS-induced MC proliferation. C57Bl/6J and OR-specific knockout (KO) mice were treated for up to 6 weeks with PBS, MS (0.7-2.14 mg/Kg), naloxone (equimolar to MS), or MS+naloxone (n=6 per group). Glomerular volume and expression of PCNA, Thy1 and ED1/CD68 were analyzed in kidney sections *in vivo*. Cell proliferation and STAT3 phosphorylation were analyzed by bromodeoxyuridine (BrdU) ELISA and Western blot, respectively, in MCs *in vitro*. MS treatment led to enlarged kidneys and glomerulopathy and naloxone reversed these effects. MS treatment increased glomerular volume in both mu-OR (MOR) KO and delta-OR (DOR) KO mice, but not in kappa-OR (KOR) KO mice. To ascertain that MS-induced glomerulopathy in vivo was due to mesangial cell proliferation, we further examined the OR-specific effects of MS in mesangial cells *in vitro*. MS-induced MC proliferation *in vitro* was inhibited by KOR-specific nor-BNI, but not by DOR or MOR-specific antagonists naltrindol or CTOP, respectively. KOR-specific agonist U50488H stimulated proliferation of MCs, but DOR-specific agonist DPDPE and MOR-specific agonist DAMGO did not. MS failed to stimulate proliferation of MCs from KOR KO mice. MS and KOR agonists induced STAT3 phosphorylation, and STAT3 inhibitor blocked KOR agonist-induced MC proliferation. We show that, MS stimulates glomerulopathy and MC proliferation via KOR and STAT3 signaling.

**Key Words**: kidney, STAT3, nephropathy
INTRODUCTION

Opioids are a mainstay of treatment for pain associated with a variety of acute and chronic disease processes. While the untoward renal effects of non-opioid analgesics are well-known (11,19), the adverse effects of chronic opioid exposure are largely unknown, with the exception of the inadequately defined entity of heroin nephropathy, which many experts believe to be due to a contaminant and not heroin itself (1,14,16,29,41,42). Recent studies from our laboratory and others have shown that chronic treatment with MS leads to kidney pathology, proteinuria, elevated urinary creatinine, increased cell proliferation and glomerular volume expansion in tumor-bearing C3H mice and rats (4,7).

Morphine sulfate (MS) is one of the most commonly used opioids and is a metabolite of heroin. There are three classical opioid receptors (OR) defined by their unique pharmacologic responses to various opioid ligands: delta (DOR), kappa (KOR), and mu (MOR) and the less well-characterized nociceptin/orphanin FQ. MS is capable of interacting with all three of the classical ORs (55). ORs, once thought to be located only in the central nervous system where they function in anti-nociception, have also been identified in the kidney (36,40). ORs appear to be functionally important in the kidney based on evidence suggesting that MS stimulates mesangial cell proliferation, migration and matrix synthesis in vitro in conditions analogous to chronic use in vivo (45,46) and promotes renal medullary interstitial cell and fibroblast proliferation (47-49). However, the cell-specific OR of importance and downstream signaling events remain unknown. As mesangial cell proliferation is a hallmark of glomerular disease, we sought in this...
study to identify the specific OR associated with MS-induced mesangial cell proliferation and the signaling pathway associated with it.

Amongst the signaling pathways, signal transducer and activator of transcription-3 (STAT3), a transcription factor, has been shown to mediate mesangial cell proliferation, epithelial tubule formation and glomerulosclerosis (9,52,59). MS and MOR agonist DAMGO stimulate STAT3 phosphorylation in differentiated SH-SY5Y neuroblastoma cells and in endothelial cells (12,56), suggesting that MS may be acting via STAT3 pathway in stimulating MC proliferation.

Therefore, we examined the effect of chronic MS treatment on mesangial cells in vivo and the specific OR and signaling pathway associated with it. To ascertain the contribution of individual ORs, we utilized an in vivo model of chronic opioid therapy in OR-specific knockout mice and in wild-type mice treated with specific OR agonists and antagonists. We show that MS stimulates mesangial cell proliferation via KOR and a STAT3-dependent mechanism.

METHODS

Reagents

Injectable MS was obtained from Baxter Esilerderle Mfd. Healthcare Corporation (Cherry Hill, NJ). D-Phe-Cys-Tyr-D-Orn-Thr-Pen-Thr-NH₂ (CTOP), norbinaltorphimine (norBNI), \([\text{D-Pen}^{2,5}]\)-Enkephalin (DPDPE), \([\text{D-Ala}^2,\text{N-Me-Phe}^4,\text{Gly}^5\text{-ol}]\)-Enkephalin
(DAMGO), and trans-(±)-3,4-Dichloro-N-methyl-N-(2-[1-Pyrrolidinyl] Cyclohexyl)-Benzeneacetamide (U50488H) were obtained from Sigma (St. Louis, MO).

Animal Models
All animal experiments complied with Institutional Animal Care and Use Committee (IACUC) approved protocols. C57BL/6J and KOR, DOR, and MOR knock-out mice produced on a C57Bl/6J background were used. Equal numbers of male and female mice were used. MOR-1 KO mice were generated by deleting exon 1 of the MOR-1 gene by using a targeted vector, in which exon 1 was replaced by a neomycin resistance cassette, as previously described (43). DOR-KO mice were produced by the deletion of exon 2 of the murine DOR-1, which was replaced by a neomycin resistance cassette (60). KOR-KO mice were produced by replacing exon 3 of the murine KOR-1 gene with the neomycin resistance cassette (26).

Opioid Treatment
Mice were treated daily with escalating doses of pharmacological grade MS (0.75, 1.4 and 2.14, 2.8, 3.6 and 4.3 mg/Kg/day in 2 divided doses during the 1st, 2nd, 3rd, 4th, 5th and 6th weeks, respectively; equivalent to about 50–301 mg/70 kg human per day). Mice treated with a KOR-specific agonist were subcutaneously injected with 1mg/kg/day of U50488H for three weeks. Mice were euthanized at the end of treatment using CO₂, and kidneys were dissected, weighed and processed for analysis.
RT-PCR

Total RNA was isolated from the kidneys using Trizol reagent. Five µg of total RNA was reverse transcribed using the first strand synthesis system (Invitrogen, Carlsbad, CA). PCRs were performed by using Taq DNA polymerase (Continental Lab Products, San Diego, CA). Sequences of primers homologous to the coding region of each gene were:
mouse MOR (GenBank acc. NM_011013): 5’-CGA CTG CTC TGA CCC CTT AG-3’ (sense nucleotides 99-118) and 5’-TCC AAA GAG GCC CAC TAC AC-3’ (antisense nucleotides 302-321); mouse KOR (GenBank acc. S77868): 5’-AGC TTG GGC AGT TGG AGT TAG TGA-3’ (sense nucleotides 919-942) and 5’-AAG CTC ACC TCC AGA TCG CTG ATT-3’ (antisense nucleotides 1237-1260); mouse DOR (GenBank acc. L06322): 5’-ATC TTC ACC CTCACC ATG ATG-3’ (sense nucleotides 264-284) and 5’CGG TCC TTC TCC TTG GAACC-3’ (antisense nucleotides 800-819); mouse GAPDH (GenBank acc. NM_001001303): 5’-CGT CTT CAC CAC CAT GGA GA-3’ (sense nucleotides 353-371) and 5’-CGG CCA TCA CGC CAC AGT TT-3’ (antisense nucleotides 635-651). To re-confirm the expression of MOR and DOR in mouse kidneys, additional primer sets were used as follows: MOR (GenBank accession #U26915) (43), 5’-AGA GGA AGA GGC TGG GGC G-3’ (sense nucleotides 195-213); 5’-CAT ACA TGA CCA GCC GTA TGG GCC TGG GGC G-3’ (sense nucleotides 531-556) and DOR (61), 5’-CTC GTC AAC CTC TCG GAC GCC-3’ (sense nucleotides 73-93) and 5’-CCT TGG AAC CGG ACA GCA GAC G-3’ (anti-sense nucleotides 757-778).

Amplification was performed for 30 cycles at 94°C for 50s, 56°C for 50s and 72°C for 50s with a final extension cycle for 10 min at 72°C in PTC-100 thermocycler (MJ Research, Waltham, MA). DNA samples were visualized by 2% agarose gel.
electrophoresis. PCR products obtained were sequenced (Microchemical Facility, University of Minnesota), to verify that they matched the expected DNA sequences.

**Histology, Collagen Staining and Immunofluorescent Microscopy:**
Formalin-fixed paraffin embedded kidney sections 4 µm thick were stained with Periodic acid-Schiff (PAS) stain and counterstained with hematoxylin. Collagen formation was determined by Masson's Trichrome staining as described by us earlier on 4 micron thick cryosections (39). For immunofluorescent analysis kidney cryosections were fixed with acetone for 10 min at room temperature and permeabilized (only for PCNA) with 0.1% Triton x 10 for 2 min on ice. Non-specific binding was blocked with 3% donkey serum and 0.5% BSA or with 1:5 dilution of mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at r.t. Sections were then incubated with a 1:200 dilution of rabbit anti-mouse PCNA or anti-mouse CD68 or mouse anti-mouse Thy1 (all from Abcam, Cambridge, MA) for 60 min at 37°C; followed by washing and staining with 1:100, anti-rabbit antibody conjugated with Texas Red or anti-mouse antibody conjugated with Rhodamine red (Jackson Immunoresearch Labs, West Grove, PA) for 45 min at r.t. For nuclear co-localization sections were incubated with a 1:10,000 dilution of DAPI (Molecular Probes, Eugene, OR) for 10 min at r.t. Immunofluorescent signals were acquired using Olympus 1X70 microscope fitted with an Olympus DP70 digital camera. Negative controls were stained using mouse or rabbit IgG instead of 1° antibodies.
Morphometric Analysis

Morphometric analysis was performed on PAS-stained kidney sections (4 µm thick) based on the method of point counting as described before (35). For measurement of mean glomerular volume (MGV), a grid containing a tessellation of points 6.0 mm apart was used. The MGV was defined as follows: MGV = \( \left( \frac{P}{A} \right)^{3/2} \times \frac{B}{k} \), where \( P \) is the average number of points per profile, \( A \) is the area in square micrometers represented by each point (\( A = 1,481 \) at the magnification used), \( B \) represents a correction factor (1.38) that assumes glomeruli were spherical, and \( k \) represents a correction factor (1.10) that assumes the variation in glomerular volume has a coefficient of variation of 10%. Blood vessels were excluded from this analysis. Glomerular number was determined in paraffin embedded, PAS stained kidney sections as described (54).

Western Blot Analysis

Lysates (40µg protein) were resolved on 3-15% gradient SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA). For immunoblotting, we used the following 1° antibodies: rabbit anti-mouse Thy1, 1:200 dilution (Santa Cruz); rabbit anti-mouse GAPDH, 1:10,000 dilution (Sigma); rabbit anti-mouse phospho-STAT3, 1:500 dilution (Santa Cruz) and anti-STAT3, 1:500 (Cell Signaling, Beverly, MA). The immunoreactive proteins were visualized using anti-rabbit secondary antibody linked with alkaline phosphatase (Amersham Life Sciences, Buckinghamshire, UK) and the ECF Western Blotting system (Amersham Life Sciences, Buckinghamshire, UK), and chemiluminescent signals were acquired using Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Densitometric analysis of
protein bands was performed using Molecular Analyst Software (Molecular Biosciences Group, Hercules, CA).

**Culture of Mesangial Cells**

Kidneys were removed from euthanized mice, and the cortex was minced according to well-established methods (33). Glomeruli were isolated through sequential sieving of tissue, and then underwent 0.1% collagenase treatment at 37°C. Glomeruli were then plated in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA), L-Glutamine (10 mM), HEPES (10 mM), insulin-transferrin-selenium (1%, Sigma), Penicillin (100 U/ml) and Streptomycin (100 U/ml) at pH 7.4. Glomeruli were then incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Outgrowths of cells occurred at 36-48 hrs. Primary culture was trypsinized for subculture after 7-10 days. Mesangial cells were negative (>99%) for endothelial (von Willebrand’s factor and αvβ3) and podocyte (podocallyxin) markers (data not shown) as determined by FACS and immunofluorescence microscopy.

**Proliferation Assay**

Proliferation of mesangial cells was determined by using a BrdU incorporation assay (Roche Diagnostics, Indianapolis, IN) (62) per the manufacturer’s instructions. Briefly, 5000 mesangial cells/well were seeded on a 96-well plate in 20% FBS containing mesangial cell medium. Cells were allowed to adhere to the plate overnight and then incubated with 1% FBS containing medium overnight. Cells were then treated with OR-specific agonists, antagonists and/or MS for 48 hours as indicated. BrdU (incorporated
during DNA synthesis) was added to the medium for the final 8 hours of treatment. Cells were incubated for 30 min with diluted peroxidase conjugated anti-BrdU antibody. Absorbance was assessed at 370 nm with 492 nm as the reference wavelength utilizing a microplate ELISA reader. Appropriate control wells were used in each experiment.

**Glomerular Filtration Rate (GFR)**

We used the inulin clearance method to estimate GFR (13). Mice were anesthetized with a combination of Inactin (thiobutabarbitual sodium, 100 mg/kg, intraperitoneally) and ketamine (10 mg/kg). Additional doses of ketamine (5 mg/kg, intramuscularly) were administrated as required. The anesthetized mice were placed on a servo-controlled surgical table that maintained body temperature at 37°C, and a tracheostomy was performed to ensure airway protection during the procedure. The right carotid artery was cannulated with PE-10 tubing connected to PE-50 tubing for continuous measurement of arterial BP and blood sampling. The right jugular vein was catheterized with PE-10 tubing for fluid infusion. The bladder was catheterized with PE-50 tubing via a suprapubic incision for urine collections. During surgery, an isotonic saline solution containing 2.25 % albumin was infused at a rate of 0.25 µl/min/g body weight. After a 60-min equilibration period inulin infusion was started. Two 30 min-urine collection and arterial blood samples were obtained for evaluation of inulin clearance. Inulin concentration in serum and urine were measured by using a rapid colorimetric method (24). GFR was calculated from urine inulin and plasma inulin concentrations and urine flow using the equation: GFR=(Urine concentration of inulin)(Urine volume)/ Plasma concentration of inulin. All values were calculated per gram of kidney weight.
Data and Statistical Analysis

Data are expressed as mean ± SD, except in mesangial cell proliferation studies data expressed as mean ± SE. Data were compared using ANOVA with Tukey Pairwise post-test comparisons between groups. Statistical significance was defined as p<0.05.

RESULTS

Classical ORs are Present on Mesangial Cells

OR activity has been suggested in the kidney based on the identification of opioid binding sites and opioid activity in the kidney (4,36,40). However, the physical expression of ORs in the kidney remains unknown. Therefore, we first examined for the expression of MOR, DOR and KOR in the kidneys of WT, MOR-KO, DOR-KO and KOR-KO mice and in the mesangial cells. RT-PCR analysis of whole kidney and mesangial cell RNA revealed the expression of KOR and MOR (Figure 1A), but the expression of DOR was barely detectable in both kidney and mesangial cells. We next examined the expression of ORs in the kidney of OR-KO mice using an additional primer set for MOR and DOR each. MOR and KOR were expressed but DOR was not expressed in WT mouse kidney (Fig 1B). MOR-KO mice only expressed KOR, KOR-KO mice only expressed MOR, and neither expressed DOR, further supporting our initial observations (Fig 1A) that DOR is not expressed in the mouse kidney. Based on our RT-PCR findings and other studies that have identified tissue and cell-specific ORs (17,51,58), we next examined the effect of MS treatment on mouse kidney in vivo.
Morphine Stimulates Kidney Growth

For *in vivo* studies mice were treated with MS for 3 weeks to simulate the effect of chronic use of MS on the kidney. Mice treated with MS and/or other OR agonists and antagonists appeared behaviorally similar to wild-type controls treated with PBS. While MS treatment did not show any significant effect on the body weight of wild-type mice, it resulted in increased kidney weight (0.191 ± 0.01 vs. 0.143 ± 0.006 g, p<0.001) and fractional kidney mass (Table 1). Treatment with naloxone antagonized the effects of MS on kidney weight (0.139 ± 0.003 vs. 0.191 ± 0.01 g, p<0.01) as well as fractional kidney mass, suggesting that the effect of MS in the kidney is OR-specific. Interestingly, naloxone treatment decreased kidney weight as well as fractional kidney mass compared to PBS (0.117 ± 0.007 vs. 0.143 ± 0.006 g, p=0.05), raising the possibility of a critical role of ORs in maintaining normal kidney phenotype.

Morphine Stimulates Glomerulopathy

Given the dramatic increase in kidney growth with MS exposure, we examined the kidneys of MS-treated mice histopathologically. MS treatment resulted in glomerular enlargement and increased mesangial area and cellularity, but tubulo-interstitial and vascular lesions were not seen (Fig 2A); these observations are consistent with our previous histopathological findings in tumor-bearing C3H mice (4). Three weeks of treatment with MS and the KOR agonist U50,488H resulted in a significant increase in glomerular volume (132mm$^3$ ± 11 and 143 ± 14, respectively, vs. 99mm$^3$ ± 10; p<0.001) (Fig 2B). Naloxone antagonized the effects of MS exposure on glomerular volume (105mm$^3$ ± 9 vs. 132mm$^3$ ± 11, p<0.001) (Fig 2B). Glomerular volume in mice exposed
to three weeks of naloxone was not statistically different than exposure to PBS (90mm$^3$ ± 8 vs. 99mm$^3$ ± 10, p>0.05). We did not observe any difference in glomerular number in kidney sections between MS and PBS groups (PBS = 124.7 ± 17.3; MS = 121.8 ± 15.5; p>0.05; n = 6 sections/kidney from 3 different mice). Therefore, glomerular enlargement does not appear to be a compensatory mechanism to substitute for reduced number of glomeruli.

Furthermore, morphine-induced increase in glomerular volume was time-dependent (Table 2 and Fig 2A). Treatment of mice with morphine for 2 days did not show an increase in glomerular volume (PBS vs morphine, p>0.05), suggesting that acute treatment does not influence glomerular pathology. However, on d10 there was a 10% increase in glomerular volume, but was statistically not significant (PBS vs morphine, p=0.06). Glomerular volume continued to increase with time from 10 d to 3 wks and upto 6 wks, last period of observation (p<0.05 for MS treatment, 10d vs 3wks and 3 wks vs 6 wks). Six weeks of treatment also showed tubular hypertrophy, suggesting that glomerular changes precede tubular pathology with morphine treatment (Fig 2A, bottom right panel).

**Morphine stimulates mesangial expansion and cell proliferation in mouse kidney**

MS stimulates mesangial cell proliferation *in vitro*. Increased mesangium can lead to glomerular volume expansion. Therefore, we examined if MS treatment leads to an increase in mesangium in mouse kidneys *in vivo* using Thy1 staining as a marker for MCs. Immunofluorescent staining for Thy1 showed increased red fluorescence for
Thy1 around the blue nuclei in the glomeruli of MS treated as compared to PBS treated mice, suggestive of increased MCs in a mesangial staining pattern (Fig. 3A-D). Enlarged glomeruli were evident at both low and high magnification in MS as compared to relatively smaller glomeruli in PBS treated animals. Western immunoblotting showed a 30% increase in band density for Thy1 in MS treated as compared to PBS treated mouse kidney lysates (Fig. 3D). It therefore appears that MS stimulates MC proliferation in vivo in the glomerulus. Staining for PCNA, an antigen that is associated with DNA synthesis during cell proliferation, revealed an appreciable amount of red staining co-localized with the nuclear stain DAPI after 3 weeks of morphine treatment, that increased further after 6 weeks of treatment, whereas no staining was observed after PBS treatment (Fig. 4A-F). Increased PCNA staining together with the observed increase in Thy1 staining for MCs after 3 weeks of treatment suggests that MS stimulates MC proliferation in vivo. PCNA is also important in DNA repair in case of injury, but glomerular pathology did not show any signs of injury, suggesting that increased PCNA is likely to be associated with cell proliferation. Increased PCNA staining may also be due to increased proliferation of other glomerular cells in addition to MCs. Since infiltrating macrophages can result in increased cytokine secretion leading to increased MC proliferation, we next examined if MS treatment influenced macrophage infiltration. Immunofluorescent staining for ED1/CD68, a macrophage-specific marker showed no appreciable difference between MS and PBS treated mice (Fig. 4G and H). Further, Masson’s Trichrome staining did not show any difference in staining between PBS and MS (results not shown). Taken together with in vitro data,
these *in vivo* observations suggest that MS stimulates MC proliferation and mesangial expansion directly leading to the expansion of glomerular volume.

Since glomerular enlargement is known to precede glomerulosclerosis, a feature of advanced kidney disease, we further examined if a specific OR mediates the activity of MS.

**Morphine Stimulates Glomerulopathy via KOR**

We next treated MOR, DOR, and KOR knock-out mice for three weeks with MS to determine the contribution of each receptor in mediating MS-stimulated glomerulopathy (Figure 2C). MS treatment resulted in increased glomerular volume in MOR knock-out (161mm$^3$ ± 13 vs. 115mm$^3$ ± 22, p<0.001) and DOR knock-out (147mm$^3$ ± 14 vs. 124mm$^3$ ± 10, p<0.001) mice compared to PBS. However, MS failed to stimulate increased glomerular volume in KOR knock-out mice compared to PBS (141mm$^3$ ± 10 vs. 138 mm$^3$ ± 12, p>0.05). These data suggest that MS stimulates glomerular-specific alterations via KOR.

**Effect of Morphine Treatment on Kidney Function**

We speculated that structural changes in the glomerulus would be accompanied by alteration in renal function. Indeed, we recently showed that MS-induced glomerulopathy was accompanied by increased blood flow in the kidney and decreased mean arterial pressure in tumor-bearing C3H mice (4). Therefore, we measured the GFR as measured by inulin clearance in wild-type mice after 3 weeks of treatment with
MS. In this study, we did not observe a significant difference in GFR after MS treatment in C57/BL6 mice (3032 ± 403 vs 3392 ± 1666 µl/min for PBS; p>0.05) (Fig. 5). This lack of a change in renal function in MS-treated mice could be due to the strain of mouse and/or the techniques used. In the present study, mice were anesthetized for a significant period of time, and anesthetics may influence renal hemodynamics via the sympathetic nervous system (37). Therefore, it is likely that the effect of MS was masked due to the anesthesia used in this study.

Morphine Stimulates Proliferation of Mesangial Cells *in vitro* via KOR

The above observations demonstrate that MS acts via KOR in stimulating glomerulopathy *in vivo*. The glomerulus is largely comprised of mesangium and earlier studies have shown that MS stimulates MC proliferation and migration (45,46). The expression of MC marker proteins such as smooth muscle actin is variable and disease dependent and may therefore not be an accurate predictor of mesangial cell specific alteration in vivo especially while analyzing the effect of MS that leads to an altered phenotype. Therefore, we examined whether MS acts directly on MC via KOR using MC *in vitro*. MS increased proliferation of mouse mesangial cells *in vitro* after 48 hours of incubation as compared to PBS (O.D. values, 2.19 ± 0.20 vs. 0.836 ±0.24, p<0.01), which was antagonized by naloxone (O.D. values, 0.92 ± 0.18 vs. 2.19 ± 0.20, p<0.01), suggesting an OR-mediated event (Fig. 6A). Furthermore, we stimulated MC with OR-specific agonists as well as MS in the presence of OR-specific antagonists. The DOR-specific agonist DPDPE and the MOR-specific agonist DAMGO did not stimulate mesangial cell proliferation compared to PBS (O.D values, 1.085 ± 0.23 and 0.871 ±
0.19, respectively, vs. 0.836 ±0.24; p>0.05). However, the KOR-specific agonist U50,488H stimulated mesangial cell proliferation compared to PBS (O.D values, 1.99428 ± 0.22 vs. 0.836 ±0.24, p<0.01), suggesting that KOR may be associated with MS-induced mesangial cell proliferation. Moreover, MS-induced mesangial cell proliferation was inhibited by the KOR-specific antagonist nor-BNI compared to PBS (O.D values, 1.033 ±0.26 vs. 2.19 ± 0.20, p<0.01), but not by either the DOR-specific antagonist naltrindol (1.929 ±0.25 vs. 2.19 ± 0.20, p>0.05) or the MOR-specific antagonist CTOP (O.D values, 1.981 ±0.20 vs. 2.19 ± 0.20, p>0.05). Furthermore, MS failed to stimulate proliferation of mesangial cells isolated from KOR KO mice (O.D values, 0.964 ± 0.29 vs. PBS, 0.836 ±0.24, p>0.05) (Fig. 6B). Taken together, these data support our in vivo observations on MS-induced glomerulopathy in knockout mice and indicate that KOR mediates MS-induced MC proliferation.

Opioids Stimulate Phosphorylation of STAT3 via KOR in Mesangial Cells

STAT3 signaling is associated with glomerular disease. We have observed previously that MS stimulates STAT3 signaling in endothelial cells (12). Given that opioids stimulate proliferation of mesangial cells, we examined whether STAT3 in mesangial cells was activated by MS and OR-specific agonists. We observed that MS and the KOR-specific agonist U50,488H stimulated STAT3 phosphorylation in a time-dependent manner (Fig. 7A). Neither the MOR agonist DAMGO nor the DOR agonist DPDPE stimulated an appreciable level of STAT3 phosphorylation. Thus, it is likely that MS-induced MC proliferation is mediated via KOR through the STAT3 signaling pathway.
Mesangial Cell Proliferation dependent upon Opioid-induced STAT3 phosphorylation

Given that opioids stimulate STAT3 signaling, as well as proliferation of mesangial cells, we next examined whether KOR agonist-induced mesangial cell proliferation was dependent upon STAT3 activation. (Fig. 7B) Mesangial cells stimulated with MS (1µM) (O.D values, 2.19 ± 0.20 vs. 0.836 ±0.24, p<0.01) or U50488H (1µM) (O.D values, 1.99428 ± 0.22 vs. 0.836 ±0.24, p<0.01) for 48 hours had enhanced BrdU incorporation compared to a medium control. This effect was inhibited in the presence of the STAT-3 inhibitor PpYLKTK-mts (0.2mM) in mesangial cells stimulated with MS (O.D values, 0.958± 0.28 vs. 2.19 ± 0.20, p<0.01) or with U50488H (O.D values, 0.918 ± 0.29 vs. 1.99428 ± 0.22, p<0.01). Together, these data show that MS stimulates MC proliferation via KOR and a STAT3-dependent signaling mechanism.

DISCUSSION

MS and its congeners are used to treat severe pain for which there is no other therapy. Several studies suggest that opioids and their receptors may influence renal physiology and pathology, but none have attempted to identify the specific ORs that mediate the renal effects of opioid analgesics. Detailed mechanistic studies have been hampered to some extent by the complex cellular architecture of the kidney, the role of multiple ORs in the kidney and the influence of both central and local mechanisms regulating renal function and dysfunction. MS has been suggested to be involved directly in the stimulation of glomerular-specific alterations and mesangial cell proliferation. Early glomerular changes can lead to end-stage renal disease. Therefore, we aimed to
identify the OR that mediates the effect of MS in MCs using in vivo models of chronic opioid therapy and OR-specific knockout mice.

We observed that chronic MS treatment induced an increase in kidney weight and glomerular volume in C57/BL6 WT mice; and mesangial cell proliferation in culture. These findings are consistent with earlier in vitro observations on MS-induced MC proliferation (45) and MS-induced glomerular volume expansion and cellular proliferation in vivo in C3H mice (4). Increased kidney size is known to be an early pathologic indicator of several chronic kidney diseases including diabetic nephropathy and sickle cell nephropathy (2,6,8,44). Increased glomerular size is also associated with increased risk of end-stage renal disease in young adults (3,18). In addition to increased kidney size and glomerulopathy, mesangial cell proliferation is associated with the development and progression of kidney disease (25,32,44).

Our in vivo observations showing increased Thy1 staining in kidneys of MS treated mice are suggestive of mesangial expansion. Since there was no appreciable difference in ED1/CD68+ cells, increased PCNA staining in the glomeruli of MS treated mouse kidneys is suggestive of increased mesangial cell proliferation. On the other hand, the apparent lack of matrix expansion upon histopathological examination as well as Trichrome staining in MS-treated mice appears to be related to observations showing that glomerular cell turnover and ECM synthesis are not closely linked in mice (23). In some rat models, however, an increased cell turnover rate and ECM synthesis were partially or closely correlated (27,28), but the increase in kidney size was not attributed
to interstitial fibrosis or an infiltrative process, as there was no light microscopic
evidence to suggest this. Given that glomeruli make up a small portion of the total
kidney volume, opioids likely stimulate other events, such as tubular and interstitial cell
proliferation. Indeed our histological observations are suggestive of tubular hypertrophy
after longer period of treatment (6 weeks), suggesting that glomerulopathy precedes
other changes induced by morphine treatment. Tubular pathology was observed by us
in C3H mice treated with morphine for 3 weeks (9), suggesting that C57 strain used in
this study may be resistant to morphine treatment early on. Our in vivo observations
are further supported by in vitro observations on MS-induced MC and renomedullary
interstitial cell and kidney fibroblast proliferation (45,47-49).

Moreover, the possibility that opioid-exposure may lead to enlarged kidneys and
subsequent hyperfiltration-mediated injury, as is seen in diabetes and sickle cell
disease, is of concern in opioid-based treatments of pain. Glomerulopathy is associated
with the development of secondary focal and segmental glomerulosclerosis (25,32,44).
Increasing evidence suggests that genetic factors play a critical role in the development
of glomerular disease (10,15). For example, C57/BL6 mice were shown to be
glomerulosclerosis resistant vs ROP mice, which were prone to glomerulosclerosis
(15,23,30). The oligosyndactyl (Os) mutation and streptozotocin treatment in both ROP
and C57/BL6 mice led to glomerular volume expansion and mesangial cellular
proliferation, but only ROP mice developed sclerosis. Interestingly, in ROP mice
glomerular volume continued to increase, whereas, it plateaued in C57/BL6 mice.
Similarly, we observed that even though MS treatment lead to an increase in glomerular
volume, it did not result in any other pathological lesions or alteration in GFR or proteinuria in our study using the C57/BL6 strain. In contrast, in a recent study on tumor-bearing C3H mice, we observed that 3 weeks of MS treatment lead to an increase in glomerular volume, PCNA expression, tubular dilatation, peri-tubular congestion and tubular casts (4), and these MS-induced histopathological changes were accompanied by proteinuria, increased blood flow in the kidneys and decreased mean arterial pressure, even though these mice did not show any metastasis in the kidney or other changes due to the growth of subcutaneous tumor. Differences in the presentation of renal disease in MS treated C3H mice described by us earlier and in C57/BL6 mice in this study suggest that genetic factors are critical to the development of MS-induced nephropathy. Although no well controlled studies are available on drug users, the available studies show membranoproliferative glomerulonephritis in white users and focal segmental glomerulosclerosis in black heroin users (1,14,16,29,41,42). The heterogeneity in response to heroin is speculated to be due to variability in socioeconomic background, viral infections and paucity of prospective controlled studies, in addition to differences in genetic susceptibility.

It is believed that mesangial proliferation occurs in response to increased cytokines secreted by the infiltrating macrophages in the mesangium. Since MS treatment did not show an increase in macrophages, the increased population of mesangial cells in MS treated mouse kidneys appears to be due to a direct effect of MS on mesangial cell proliferation. Our data therefore argue for MS-induced mesangioproliferative glomerulopathy in this mouse model.
We therefore sought to identify the OR associated with MS activity in mesangial cells, using C57/BL6 mice deleted for ORs. Since C57/BL6 mice develop mesangial and glomerular volume expansion which precedes more severe glomerular disease in humans, it appeared to be a suitable strain to identify the OR associated with early glomerular disease initiated by MS.

We found in the present study that MOR and KOR were expressed in the kidney as well as MCs in vitro. The MS-induced increase in glomerular volume and mesangial cell proliferation appeared to be predominantly associated with KOR. It is interesting to note that earlier studies from our laboratory and others have shown that MS via MOR stimulates endothelial proliferation and angiogenesis in vitro and in tumors and healing wounds (22,39,50). However, in the present study histopathological examination of MS treated mouse kidneys did not suggest any vascular alteration. Taken together previous studies and the present investigation suggest that MOR mediates endothelial activity while KOR mediates mesangial cell activity. Indeed, our data consistently indicate that KOR mediates MS-induced glomerulopathy and MC proliferation in vivo and in vitro, based on a confluence of evidence from OR-specific knockout mice, OR-specific agonism and OR-specific antagonism of MS-induced effects. Exogenous and endogenous opioids and KOR have been shown to influence renal physiology, including water retention (20,34). KOR agonists have been shown to induce water diuresis in rats, by an as yet unknown mechanism (57). High levels of b-endorphins have been observed in patients with chronic renal failure on dialysis (5). This increase in endogenous opioids has been potentially linked to uremic pruritis which was
ameliorated over the short-term by the use of a non-selective OR antagonist naltrexone in a clinical trial (38). While these studies raise the possibility of both central and local effects of opioids on renal physiology, our observations show the direct effect of MS on glomerular-specific alterations that are mediated by KOR. This finding is critical to the development of new approaches to analgesic therapy that will provide analgesia without promoting renal disease.

In addition, we show that MS-induced mesangial cell proliferation via KOR appears to be dependent upon activation of STAT3. We have shown recently that MS stimulates STAT3 signaling in endothelium via MOR (12), and others have shown that DOR activation leads to STAT3 phosphorylation (31). Furthermore, MS-induced cardioprotection is dependent upon STAT3 phosphorylation in rat heart and H9C2 cardiac myoblasts (21). It is known that STAT3 phosphorylation participates in the pathogenesis of glomerulosclerosis in an experimental model of glomerulonephritis induced by anti-Thy 1.1 antibodies in rats (53). From these observations it appears that STAT3 plays a critical role in the pathological processes in the kidney and that different opioid receptors can stimulate STAT3 signaling in a tissue- and cell-specific manner. These data, therefore, support our observations that MS-induced STAT3 signaling may be critical in pathogenesis of glomerular disease. Moreover, the STAT3 inhibitor peptide PpyYKTK-mts counteracted MS- and KOR agonist-induced MC proliferation. Based on these findings, we speculate that STAT3 may be an additional target for developing combination therapy with STAT3 inhibitors and opioid analgesics. Such combination
therapy would in theory prevent inadvertent glomerular disease, if in fact further investigations confirm our findings that opioids are active in the kidney.

In conclusion, we demonstrate that chronic MS treatment stimulates time-dependent increase in glomerular volume and mesangial cell proliferation suggestive of diffused mesangioproliferative glomerulonephropathy in the mouse model studied. MS acts directly on MCs and stimulates their proliferation via KOR and STAT3 signaling. We speculate that chronic use of opioids may lead to the development or exaggeration of renal disease. On the other hand, our study identifies targets including KOR and STAT3, for developing novel therapeutics to prevent and treat renal disease.

Acknowledgements

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43. Schuller AGP, King MA, Zhang J, Bolan E, Pan YX, Morgan DJ, Chang A, Czick ME, Unterwald EM, Pasternak GW, Pintar JE. Retention of heroin and


FIGURE LEGENDS

Figure 1. (A) DOR, KOR, and MOR expression in mouse kidneys and mesangial cells by RT-PCR. Primer sets used were: mouse MOR (GenBank acc. NM_011013): 5'-CGA CTG CTC TGA CCC CTT AG-3' (sense nucleotides 99-118) and 5'-TCC AAA GAG GCC CAC TAC AC-3' (antisense nucleotides 302-321); mouse KOR (GenBank acc. S77868): 5'-AGC TTG GGC AGT TGG AGT TAG TGA-3' (sense nucleotides 919-942) and 5'-AAG CTC ACC TCC AGA TCG CTG ATT-3' (antisense nucleotides 1237-1260); mouse DOR (GenBank acc. L06322): 5'-ATC TTC ACC CTCACC ATG ATG-3' (sense nucleotides 264-284) and 5'CGG TCC TTC TCC TTG GAACC-3' (antisense nucleotides 800-819); mouse GAPDH (GenBank acc. NM_001001303): 5'-CGT CTT CAC CAC CAT GGA GA-3' (sense nucleotides 353-371) and 5'-CGG CCA TCA CGC CAC AGT TT-3' (antisense nucleotides 635-651). (B) OR expression in kidneys of MOR-KO, DOR-KO and KOR-KO mice. Two different primer sets were used for the expression of MOR and DOR. Results shown here were observed with KOR primers described in 1A, MOR primers, 5'-AGA GGA AGA GGC TGG GGC G-3' (sense nucleotides 195-213); 5'-CAT ACA TGA CCA GGA AGT TTC CAA AG-3' (anti-sense nucleotides 531-556) and DOR primers, 5'-CTC GTC AAC CTC TCG GAC GCC-3' (sense nucleotides 73-93) and 5'-CCT TGG AAC CGG ACA GCA GAC G-3' (anti-sense nucleotides 757-778); n= RNA from 3 different mouse kidneys or mesangial cell cultures; and mouse brain RNA from WT mice was used as positive control (A and B).
**Figure 2.** (A) Effect of morphine treatment on histology in PAS-stained kidney sections. Morphine induces a time-dependent increase in glomerular volume and in cell number in the glomerulus seen as increased number of purple nuclei, after 3 and 6 weeks of treatment. Magnification 630X. (n = 6), Scale bar = 20 µm (B) Quantitation of glomerular volume in PAS-stained sections of kidneys of three-month-old WT mice exposed to three weeks of PBS, naloxone, MS, MS + naloxone, or the KOR agonist U50,488H. MS exposure led to an increase in the glomerular volume compared to PBS, which was antagonized by naloxone. Each bar represents glomerular volume determination in 50 glomeruli/mouse from 6 different mice. *p<0.001 vs PBS, †p<0.001 vs MS. (C) Glomerular volume in MS- or PBS-treated OR-specific knockout mice. MS stimulated increased glomerular volume in MOR KO and DOR KO mice compared to PBS. However, MS failed to stimulate glomerular volume in KOR KO; n = 50 glomeruli/mouse from 6 different mice. ‡p<0.001 vs MOR KO PBS, §p<0.001 vs DOR KO PBS. Results are expressed as mean ± SD.

**Figure 3.** Effect of 3 weeks of MS treatment on Thy1 expression in kidney. Whole kidney cryosections or lysates from mice treated with PBS or MS for 3 weeks were stained / immunoblotted using antibodies to mesangial cell marker Thy1, as described in the methods. Double immunostaining using DAPI for nuclear colocalization in blue and red staining for Thy1 around the nuclei, shows increased red staining in MS as compared to PBS under both low (A,B) and
Figure 4. Effect of MS treatment on PCNA and CD68. Kidney cryosections from mice treated with PBS or MS for three-six weeks, were immunostained with cell proliferation marker PCNA antibody or anti-CD68 and co-stained with DAPI for nuclear co-localization. Red staining in the mesangial staining pattern in the nuclear region is suggestive of cell proliferation in the glomerulus of MS (B) and (C) but no staining is seen in PBS (A). Co-stained nuclei with DAPI for A, B and C are shown in D, E and F respectively. Cytoplasmic staining for the macrophage marker CD68 in red surrounds blue nuclei (G,H). Scale bar, A-F = 100 µm. N = 3 for each staining.

Figure 5. Effect of MS on renal function. GFR expressed in µL/min in mice treated with MS or PBS for three weeks as determined using the inulin clearance method. n=6 mice per treatment. Results are expressed as mean ± SD.

Figure 6. Effect of opioids on mesangial cell proliferation in vitro. (A) MC were isolated from C57/BL6 WT mice and treated with 1 µM each of MS and/or OR-specific agonists and antagonists for 48 h as indicated. Cell proliferation was
quantitated using BrdU ELISA. n=6. p<0.01 for *PBS vs MS or U50488H, p<0.01 for †MS vs MS with Naloxone or MS with nBI. (B) Effect of MS on MC isolated from KOR-KO mice after 48 h of incubation. n= 6. Results are expressed as mean ± SEM.

**Figure 7.** (A) MC from WT C57/BL6 mice were stimulated with 1 µM each of MS or OR-specific agonists as shown above for different time periods. Expression of phospho-STAT3 and total STAT3 was determined by Western immunoblotting. The ratio of phospho-STAT3 and STAT3 band density is shown numerically above each set of bands. Each blot represents 3 similar and reproducible experiments using MCs derived from 3 different mice. (B) Mesangial cells from WT C57/BL6 mice were stimulated with 1 µM each of MS or U50488H for 48 hours in the presence or absence of the STAT3 inhibitor peptide, PpYLKTK-mts (0.2mM), followed by BrdU incorporation ELISA to quantitate cell proliferation. n = 6 experiments per treatment group. Results are expressed as mean ± SEM. p<0.01 for *PBS vs MS or U50488H; p<0.01 for †MS vs MS + PpYLKTK mts; p<0.01 for §U50488H vs U50 + PpYLKTK.
Table 1. Kidney and Body Weights and Age of the Mice

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>Kidney Weight (in g ± SD)</th>
<th>Mouse Weight (in g ± SD)</th>
<th>Kidney:Body Weight Ratio</th>
<th>Age (in months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT PBS</td>
<td>0.290 ± 0.01</td>
<td>22.6 ± 2.7</td>
<td>1.28 ± 0.25</td>
<td>~ 4</td>
</tr>
<tr>
<td>WT MS</td>
<td>0.382 ± 0.02</td>
<td>23.6 ± 3.4</td>
<td>*1.62 ± 0.3</td>
<td>~ 4</td>
</tr>
<tr>
<td>WT MS + Naloxone</td>
<td>0.278 ± 0.006</td>
<td>23.3 ± 2.9</td>
<td>†1.20 ± 0.3</td>
<td>~ 4</td>
</tr>
<tr>
<td>WT Naloxone</td>
<td>0.234 ± 0.01</td>
<td>21.9 ± 2.1</td>
<td>§1.07 ± 0.2</td>
<td>~ 4</td>
</tr>
</tbody>
</table>

WT = wild type C57/BL6 mice. Each value represents mean ± SD for 6-10 mice.

* p<0.001 WT PBS vs WT MS
† p<0.01 WT MS vs WT MS + Naloxone
§ p≤0.05 WT PBS vs WT Naloxone
Table 2. Time-dependent increase in glomerular volume in wild type mice treated with morphine

<table>
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<tr>
<th>Treatment</th>
<th>Number of Days</th>
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</tr>
<tr>
<td>PBS (mm³)</td>
<td>96 ± 10</td>
</tr>
<tr>
<td>Morphine (mm³)</td>
<td>98 ± 9</td>
</tr>
</tbody>
</table>

Mice were treated with escalating doses of morphine 0.75, 1.4, 2.14, 2.8, 3.6 and 4.3 mg/kg morphine per day for the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> week, respectively.

Each value represents mean ± SD from 50 glomeruli/mouse in 5-6 mice. *p<0.001 vs PBS for 21 days; †p<0.001 vs PBS for 42 days; *p<0.05 vs morphine for 10 days; #p<0.05 vs morphine for 21 days
A

<table>
<thead>
<tr>
<th>Base pairs</th>
<th>Ladder</th>
<th>Brain</th>
<th>Kidney</th>
<th>Mesangial cells</th>
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<td>300</td>
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<td></td>
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<tr>
<td>300</td>
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</tbody>
</table>

MOR (220 bp)

KOR (342 bp)

DOR (365 bp)

GAPDH (300 bp)

B

<table>
<thead>
<tr>
<th></th>
<th>Brain</th>
<th>Kidney</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
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<tr>
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</tr>
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<tr>
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<tr>
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</table>

MOR

KOR

DOR

GAPDH
3 weeks

A

PBS

MS

6 weeks

B

C

<table>
<thead>
<tr>
<th>Glomerular Volume (mm$^3$)</th>
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<tbody>
<tr>
<td>PBS</td>
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</tr>
</tbody>
</table>

* $p < 0.001$ vs PBS
† $p < 0.001$ vs MS

<table>
<thead>
<tr>
<th>Glomerular Volume (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR KO PBS</td>
</tr>
<tr>
<td>140</td>
</tr>
</tbody>
</table>

‡ $p < 0.001$ vs MOR KO PBS
§ $p < 0.001$ vs DOR KO PBS
p<0.01 for
* PBS vs MS or U50488H
† MS vs MS with Naloxone or MS with nBNI

A

B

OD570nm

MS with Antagonists
MS with Agonists

PBS, MS, Naloxone, Naltrexone, nBNI, CTOP, DPDPE, U50488H, DAMGO

OD570nm

PBS, MS

0.25 0.5 0.75 1 1.25