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Rapid, nongenomic stimulation of multidrug resistance protein 2 (Mrp2) activity by glucocorticoids in renal proximal tubule

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Non-standard abbreviations: CAR, constitutive androstane receptor; FXR, farnesoid xenobiotic receptor; ERK, extracellular-signal-regulated kinase; ET, endothelin; FL-MTX, fluorescein methotrexate; GR, glucocorticoid receptor; HGF, Hepatocyte growth factor; MAPK, mitogen-activated protein kinase; MDCKII, Madin-Darby canine kidney strain II cells; c-Met, mesenchymal epithelial transition factor; MTS, marine teleost saline; MR, mineralocorticoid receptor; Mrp2, multidrug resistance protein 2; NHE, Na^+/H^+-exchanger; NO, nitric oxide; iNOS, inducible NO synthase; Oat, organic anion transporter; PXR, pregnane xenobiotic receptor; TRK, tyrosine receptor kinase;

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Abstract

In renal proximal tubule, multidrug resistance protein 2 (Mrp2) actively transports many organic anions into urine, including drugs and metabolic wastes. Upon exposure to nephrotoxicants or during endotoxemia, both Mrp2 activity and expression are up-regulated. This may result from induced de-novo synthesis of Mrp2 or post-transcriptional events involving specific signaling pathways. Here we investigated glucocorticoid signaling to Mrp2 in killifish renal proximal tubules, a model system in which transport activity can be measured using a fluorescent substrate and confocal imaging. Exposure of tubules to dexamethasone rapidly increased Mrp2-mediated fluorescein methotrexate (FL-MTX) transport. Other glucocorticoid receptor (GR)-ligands, cortisol and triamcinolone acetonide, also stimulated Mrp2-mediated transport. The GR-antagonist, RU486, abolished stimulation by all three ligands, whereas the mineralocorticoid receptor antagonist, spironolactone, was ineffective. Consistent with action through a nongenomic mechanism, dexamethasone stimulation of Mrp2-mediated transport was insensitive to cycloheximide and actinomycin D, and immunohistochemistry revealed no alterations in Mrp2 expression at the luminal membrane. K252a, an inhibitor of the tyrosine kinase TRK subfamily, reduced the dexamethasone effect, as did the specific c-Met receptor tyrosine kinase inhibitor, PHA-665752. Hepatocyte growth factor (HGF), endogenous ligand for c-Met, stimulated Mrp2-mediated transport. This effect was reversed by PHA-665752 but not by RU486. Inhibition of MEK1/2 also abolished the effects of dexamethasone and HGF. Our results disclose a novel mechanism by which glucocorticoids acting through GR, c-Met and MEK1/2 cause rapid, nongenomic stimulation of Mrp2-mediated transport in renal proximal tubules. This up-regulation may be nephroprotective, enhancing efflux of metabolic wastes and toxicants during cell and tissue stress.

Introduction

The multidrug resistance protein isoform 2 (MRP2/ABCC2) is expressed at the luminal membrane of vertebrate renal proximal tubules, where it drives ATP-dependent efflux of anionic xenobiotics and metabolic wastes into urine. Previous studies with rats *in vivo*, with MDCKII cells over-expressing human MRP2 and with isolated killifish (*Fundulus heteroclitus*) renal tubules showed that Mrp2-mediated transport is rapidly reduced by endothelin-1 (ET-1) acting through an ET-B receptor, inducible nitric oxide synthase (iNOS), cyclic GMP and protein kinase C (Masereeuw et al., 2000;Notenboom et al., 2004). A number of nephrotoxicants, including radiocontrast agents, aminoglycoside antibiotics and heavy metal salts, initiate the same sequence of events (Terlouw et al., 2002). In the long-term, however, luminal Mrp2 activity and protein expression are increased 24 hours after transient exposure to ET-1 or to nephrotoxicants. Blocking this efflux pump resulted in an enhancement of cell damage and necrosis, indicating that its up-regulation during toxic stress is a prerequisite for survival (Heemskerk et al., 2007). Such up-regulation may result from induced de-novo synthesis of Mrp2, or post-transcriptional regulation involving receptors and signaling pathways that alter Mrp2 function (Notenboom et al., 2005;Notenboom et al., 2006). In support of transcriptional up-regulation, increased levels of Mrp2 mRNA were found in the kidney after exposure to several toxicants and in chronic renal failure (van de Water et al., 2005).

The glucocorticoids, cortisol and corticosterone, are stress hormones involved in diverse physiological processes. These hormones exert their biological effects by binding to the intracellular glucocorticoid receptor (GR; NR3C1) or mineralocorticoid receptor (MR; NR3C2), which upon activation translocate to the cell nucleus and bind to specific DNA sequences to regulate transcription of target genes. Corticosteroid binding sites in the plasma membrane have also been detected (Orchinik et al., 1994). In general, the GR controls metabolism, immunity, and the stress response; MR regulates electrolyte homeostasis and blood pressure. Glucocorticoids and their synthetic analogs, such as dexamethasone, are used clinically as immunosuppressive and anti-inflammatory agents, and to treat diarrhea associated with inflammatory bowel disease (Lowenberg et al., 2008). However, glucocorticoids also produce serious side effects and excess of endogenous glucocorticoids is associated with obesity, hypertension, hyperlipidemia, and glucose intolerance, as observed in Cushing's syndrome (Beck et al., 2009).

In the kidney, glucocorticoids determine the acid-base balance through regulation of Na^+ and $HCO3^-$ absorption (Hulter et al., 1980). The apical expression of the most abundant Na^+/H^+-exchanger (NHE) isoform, NHE3 is up-regulated through increased transcription in response to glucocorticoids. However, a rapid non-genomic GR-initiated up-regulation that does not involve increases in protein abundance also occurs (Wang et al., 2007). Moreover, maternal treatment with dexamethasone during pregnancy reduces kidney development in the young (de Vries et al., 2010) and stimulates renal tubular NHE activity (Dagan et al., 2007), effects that possibly relate to development of hypertension in adult life.

It is not known whether other membrane transporters, including MRP2, are regulated by GR activation. In primary hepatocytes, dexamethasone increases MRP2 expression, however, this transcriptional regulatory pathway involves activation of the nuclear receptor, pregnane xenobiotic receptor (PXR; NR1I2), rather than GR activation (Kast et al., 2002). In the present study, we map a signaling pathway in renal proximal tubule by which glucocorticoids rapidly increase Mrp2 transport activity. For these experiments, we used isolated renal proximal tubules from killifish and confocal microscopy. Our results show that dexamethasone targets Mrp2 through a non-genomic mechanism, involving GR, the receptor tyrosine kinase, cMet, and MEK/ERK 1/2. This novel signaling pathway may serve to enhance efflux of accumulating wastes during cell and tissue stress.

Materials and methods

Chemicals. Fluorescein methotrexate (FL-MTX) and Alexa Fluor 488-labeled goat anti-mouse IgG were obtained from molecular probes (Eugene, OR, USA). Mouse monoclonal M2III-6 antibody to Mrp2 was from Alexis-Axxora (San Diego, CA, USA). Actinomycin-D, 15α-androstan-17β-ol, bisindolylmaleimide (BIM), chenodeoxycholic acid, clotrimazole, cortisol, cortisone, cycloheximide, dexamethasone, dehydroepiandrosterone, HGF, LY-294002, NG-methyl-L-arginine, mifepristone (RU486), n-propyl p-hydroxybenzoate, 5β-pregnane-3,20-dione, pregnenolone-16α-carbonitrile, triamcinolone acetonide, U0126, SB203580 and modified medium 199 with Earle's salts were purchased from Sigma Chemicals (St. Louis, MO, USA). Genistein, K252a and PHA-665752 were obtained from Calbiochem (San Diego, CA, USA). All other chemicals used were obtained at the highest purity available.

Animals. All animal studies were performed in accordance with institutional regulations for animal protection. Killifish (*Fundulus heteroclitus*) were wild-caught in the vicinity of Mount Desert Island, Maine. The fish were maintained in tanks with recirculating natural seawater at the Mount Desert Island Biological Laboratory (MDIBL), experiments were performed in accordance with legislation in Maine (USA).

Transport experiments. Killifish renal (proximal) tubules were used as model for two reasons. First, renal tubules of fishes have, in contrast to mammalian renal tubules, the capacity to reseal after isolation; they form a closed compartment and are still viable for transport studies. Second, killifish tubules are unique since they have a high Mrp2 expression in proximal tubules; the dissected and teased tubules reseal quickly; transport closely resembles transport processes characterized in humans (Masereeuw et al., 1996;Miller et al., 1996). Experiments were carried out at room temperature (20–22°C). For an experiment, 4-6 fish were killed by decapitation and pooled renal tubular masses were collected and transferred into a petridish filled with marine teleost saline (MTS), containing 140 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl2, 1.0 mM MgCl2 and 20 mM Tris at pH 8.0. Under a dissecting microscope the kidney was teased with fine forceps to remove adherent hematopoietic tissue. For a first screening experiment, renal tubules were transferred into a well plate, containing 2 ml modified medium 199 (M199) with Earle's salts supplemented with 30 mM NaCl, 4.2 mM NaHCO3, 1.0 mM L-glutamine, 25.0 mM Hepes, 14.75 mM

NaOH (pH 7.5, 347 mOsmol/kg H2O), 20 mg/ml tetracycline, 10 µg/ml insulin and 5 µg/ml hydrocortisone (Renfro et al., 1999), without (controls) or with added chemicals. Chemicals were added from stock solutions in MTS or DMSO. The final DMSO concentration never exceeded 0.5% and did not affect FL-MTX transport (Breen et al., 2004). After 3 hours incubation at 14 °C and 95% O2/5% CO2, tubules were washed in MTS and transferred to a foil-covered chamber (Bionique, Saranac Lake, NY) containing 1 ml MTS and 2 µM fluorescent-methotrexate (FL-MTX). For all experiments except for the first screening experiment, tubules were incubated for 1 h in MTS only and were directly transferred to the chambers containing the added chemicals and FL-MTX. The chamber floor was a 4 x 4 cm glass coverslip, through which the tubules could be viewed by means of a confocal laser-scanning microscope with inverted objectives. Tubules were incubated for 1 h at room temperature, to reach steady-state distribution of FL-MTX. It was previously demonstrated that FL-MTX is not metabolically degraded when incubated with killifish proximal tubules for periods up to 1 h or more (Masereeuw et al., 1996;Masereeuw et al., 2000).

Confocal microscopy. After incubation with FL-MTX, tubules in the chambers were placed on an inverted confocal microscope (Olympus Fluoview 1000 or Zeiss LSM 510) and viewed with a x20 dry objective or x40 water immersion objective. Under transmitted light, intact tubules were selected; then confocal fluorescent images were acquired using the 488-nm line of an argon ion laser, a 510-nm dichroic filter, and a 515-nm long-pass emission filter. Four scans, 4 s each, were averaged to provide a final image (512 x 512 x 8 or 12 bits). Stored images were analyzed using ImageJ 1.43u (NIH, MD, USA), as described in detail (Miller et al., 1996;Masereeuw et al., 1996;Masereeuw et al., 2000;Notenboom et al., 2004;Notenboom et al., 2005).

Immunohistochemistry. For the analysis of Mrp2 expression at the luminal membrane, tubules of killifish were dissected and treated with 1 µM dexamethasone for 1 h as described above. After incubation with dexamethasone, whole mounts of tubules were stained for Mrp2. For this purpose, tubules were washed in MTS, fixed for 10 min at room temperature in 2% (v/v) formaldehyde/0.1% (v/v) glutaraldehyde, permeabilized for 20 min in 1% (v/v) triton X-100, and incubated for 90 min with a mouse monoclonal M2III-6 antibody to Mrp2 at 37°C, and for 60 min with the secondary, Alexa Fluor 488-labeled goat anti-

mouse IgG antibody at 37°C. For staining of the GR, tubules were incubated for 90 min with a rabbit GR antibody to trout GR (1:500) at 37°C (Bury et al., 2003), and for 60 min with the secondary, Alexa Fluor 594-labeled goat anti-rabbit IgG antibody (1:200) at 37°C. After staining, tubules were transferred to a Teflon chamber, containing MTS, and Mrp2 expression was visualized using the Olympus Fluoview inverted confocal laser scanning microscope. Acquired confocal images were analyzed as before; only now in Mrp2-stained tubules: the entire luminal membrane was traced manually and mean pixel intensity was determined for each tubule.

Data analysis. Most data are presented as a percentage of fluorescence intensity observed in controls (mean values ± S.E.M.). For statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was applied using GraphPad Prism® (version 5.02 for Windows; Graph Pad Software, San Diego, CA, USA). Means were considered significantly different when $p < 0.05$.

Results

Dexamethasone stimulates Mrp2-mediated transport in renal proximal tubules. Isolated renal proximal tubules from certain teleost fish provide a convenient model for the study of membrane transport and its regulation (Masereeuw et al., 1996;Miller and Pritchard, 1997;Miller et al., 1996). These tubules are easily isolated and long-lived when maintained in a simple physiological saline. Previous studies with killifish renal proximal tubules have established functional assays for teleost Oat, P-glycoprotein and Mrp2 (Schramm et al., 1995;Miller et al., 1996;Terlouw et al., 2001). These are based on the use of confocal microscopy and digital image analysis to measure the steady state distribution of fluorescent substrates in intact, living tubules. We previously showed that luminal accumulation of the fluorescent organic anion, FL-MTX, can be used as an indicator of Mrp2 transport activity in killifish renal proximal tubules (Masereeuw et al., 2000;Terlouw et al., 2001). Such accumulation is specific, energy dependent and concentrative (Fig. 1A).

In mammalian liver, Mrp2 expression is induced through activation of nuclear receptors, *viz.* PXR, the constitutive androstane receptor (CAR; NR1I3), and farnesoid xenobiotic receptor (FXR; NR1H4) (Kast et al., 2002). Although Mrp2 expression in the kidney can be regulated by these nuclear receptors (Bauer et al., 2008), their expression levels in kidney appear to be low (Cheng and Klaassen, 2006;Zhang et al., 1999). In initial experiments, we measured Mrp2-mediated transport in isolated killifish tubules after exposure to various potent PXR or FXR ligands in various species and in zebrafish (PXR) (Parks et al., 1999;Moore et al., 2002). FL-MTX transport was not affected by the FXR ligand, chenodeoxycholic acid, nor by the PXR ligands clotrimazole, pregnenolone-16α-carbonitril, 15α-androstan-17β-ol, n-propyl p-hydroxybenzoate, 5β-pregnane-3,20-dione, dehydroepiandrosterone (data not shown). Only dexamethasone, a PXR and GR ligand, increased Mrp2-mediated transport (Figs. 1A and 1B).

Exposing killifish tubules to 0.25-1.0 µM dexamethasone increased luminal FL-MTX accumulation in a concentration-dependent manner (Fig. 1E); at higher dexamethasone concentrations, FL-MTX accumulation tended to fall off. Dexamethasone had no significant effect on cellular FL-MTX accumulation and it did not stimulate transport of the Oat substrate, FL (data not shown). Thus, dexamethasone did not target basolateral organic anion transporters.

The greatest stimulation of Mrp2-mediated transport (118 ± 10%) occurred at 1 µM dexamethasone and this concentration was chosen for subsequent experiments. To determine the time course of dexamethasone action, we incubated tubules to steady state in medium containing FL-MTX and then added dexamethasone. Mrp2-mediated transport, as indicated by luminal FL-MTX, increased rapidly following addition of the drug, with a significant change within 10 min (Figure 1F). The increase in luminal fluorescence intensity was maximal at 30 min and remained at that level for at least 3 h (not shown). The rapid time course of dexamethasone action suggests a non-genomic signaling mechanism. In subsequent experiments focused on the mechanism of dexamethasone signaling to Mrp2, tubules were exposed to 1 µM dexamethasone for 1 h to ensure both steady state distribution of FL-MTX and maximal stimulation.

Dexamethasone stimulates Mrp2-mediated transport through GR. Dexamethasone is a potent synthetic glucocorticoid. Figure 2A shows that the GR-antagonist, RU486, abolished the effect of dexamethasone on FL-MTX transport in the renal tubules; RU-486 by itself did not alter transport. Consistent with this, the endogenous teleost fish GR ligand, cortisol, and the synthetic GR ligand, triamcinolone acetonide, increased luminal FL-MTX accumulation; their effects were also abolished by RU-486 (Figs. 1C, 2B and 2C). In contrast, the mineralocorticoid receptor (MR) antagonist, spironolactone, did not block the effects of dexamethasone (Fig. 2D). Cortisone, an inactive metabolite of the native fish GR-ligand, cortisol, did not alter Mrp2 function (Fig. 1D). Finally, with an antibody directed against rainbow trout GR (Teitsma et al., 1998), we investigated receptor expression in killifish renal proximal tubules. Immunohistochemistry shows clear expression of the GR at the basolateral and luminal plasma membranes of the tubule epithelial cells, with lower diffuse, cytoplasmatic labeling (Figure 2E). These findings indicate that dexamethasone, cortisol and triamcinolone acetonide act through a killifish GR to stimulate Mrp2-mediated transport in renal tubules.

Dexamethasone does not alter Mrp2 protein expression. Dexamethasone was recently shown to regulate the Na+/H+ exchanger 3 (NHE3) (Wang et al., 2007) and to protect the kidney from ischemic injury by GR-dependent, non-genomic mechanisms (Kumar et al., 2009). Figures 3A and 3B show that the dexamethasone-induced stimulation of Mrp2-mediated transport was not affected when tubules were pre-

incubated with actinomycin D, an inhibitor of transcription, or cycloheximide, an inhibitor of translation. Consistent with these results, immunohistochemistry revealed that Mrp2 expression in the luminal plasma membrane had not increased after incubation with dexamethasone (Figure 3C-G). In addition, treatment of tubules with the microtubule inhibitor, colchicine, did not affect the ability of dexamethasone to increase FL-MTX transport (Fig. 3H). Thus, enhanced transport activity was not due to synthesis of new transporter nor to insertion of preformed transporter protein into the luminal plasma membrane.

Dexamethasone signals through cMet, a receptor tyrosine kinase. We previously demonstrated that loss of Mrp2 activity in killifish tubules involved ET-1 binding to an ETB receptor followed by activation of iNOS and PKC (Notenboom et al., 2005;Terlouw et al., 2001). To determine whether elements of this pathway are involved in the dexamethasone-induced stimulation, we measured the effects of NG-methyl-L-arginine (NOS inhibitor) and bis-indolylmaleimide (PKC inhibitor). Neither drug altered the ability of dexamethasone to stimulate Mrp2 activity (Fig. 4). Thus, the ET-1 signaling pathway was not involved in the GR-related pathway.

GR can act through multiple kinase-based signaling pathways (Beck et al., 2009). Figure 5A shows that genistein, a non-specific tyrosine kinase inhibitor abolished the effect of dexamethasone on Mrp2-mediated transport. K252a, a kinase inhibitor of the tyrosine receptor kinase (TRK) subfamily (Tapley et al., 1992), also blocked the dexamethasone-induced effect (Fig. 5B), as did the specific c-Met kinase inhibitor, PHA-665752 (Christensen et al., 2003), (Fig. 5C). These results support the involvement of the mesenchymal epithelial transition factor, c-Met, a TRK, in GR signaling to Mrp2 (Fig. 5C). The natural ligand for c-Met is hepatocyte growth factor (HGF), also known as scatter factor. HGF is both renotropic and nephroprotective (Vargas et al., 2000). Exposing killifish tubules to human recombinant HGF produced a rapid and potent increase in FL-MTX transport; significant stimulation of luminal FL-MTX was evident within 5 min of exposure to an optimal concentration of 25 ng/ml (Fig. 6A+B). This HGF-mediated effect was blocked by pretreatment with PHA-665752, but not by RU486; from this we conclude that HGF/c-Met signaling is downstream of GR action (Fig. 6C+D).

Activation of c-Met can initiate intracellular signaling through additional downstream phosphorylation cascades. When killifish tubules were exposed to U0126, which blocks a MAPK pathway

by inhibiting MEK1/2 (Favata et al., 1998), the effects of dexamethasone and of HGF on FL-MTX transport were abolished (Fig. 7A). No such effects were seen with LY294002, which inhibits the PI-3kinase/Akt pathway (Vlahos et al., 1994) (Fig. 7B), nor with SB203580, which inhibits Akt and p38 MAP kinase (Saklatvala et al., 1996) (Fig. 7C). These findings are consistent with dexamethasone acting through GR, cMet and then MEK/ERK 1/2 to increase Mrp2 transport activity.

Discussion

In the present study, we used killifish renal proximal tubules to map a novel signaling pathway through which glucocorticoids rapidly stimulate Mrp2-mediated transport. Three main findings substantiate this pathway. First, dexamethasone and triamcinolone acetonide, potent synthetic glucocorticoids, and cortisol, the natural fish glucocorticoid, increased Mrp2-mediated transport of FL-MTX. These effects were blocked by the GR antagonist, RU-486, but not by the MR antagonist, spironolactone. These results establish GR as the receptor through which these glucocorticoids activate Mrp2. Glucocorticoids are released in response to physiological stress, eliciting GR-specific responses in multiple if not all cell types. Signaling by GR can be complicated, involving multiple receptor subtypes and both genomic and non-genomic actions. Rapid glucocorticoid effects generally involve cytosolic GRs, although GRs can also act within membranes to exert multiple rapid effects on various tissues and cells, as is described in teleost and higher vertebrates (Borski, 2000;Roy and Rai, 2009). More specifically, membrane-bound GRs were found to have analogous ligand binding domains (epitope recognition, ligand specificity and phosphorylation site) comparable to cytosolic GRs (Bartholome et al., 2004;Gametchu et al., 1999) and RU486 does not discriminate between the GR subtypes (Borski, 2000). We show here that killifish renal proximal tubules immunostained for GR show extensive labeling of the basolateral and luminal plasma membranes as well as less intense, diffuse intracellular labeling. At present, it is not clear whether the dexamethasone stimulation of Mrp2 activity resulted from ligand binding to membrane-bound or intracellular GR.

Second, dexamethasone stimulation of Mrp2 transport activity had a rapid onset and was not affected when transcription or translation was inhibited. Immunostaining showed no increase in Mrp2 protein expression in dexamethasone-exposed tubules. These observations indicate a non-genomic mechanism of GR regulation of Mrp2 activity. This non-genomic action of dexamethasone, involving the GR, is also in agreement with results from rat kidney where the glucocorticoid showed renoprotective effects against ischemia reperfusion injury (Kumar et al., 2009). In addition to our results with killifish renal tubules, Mrp2 gene expression in zebrafish did not increase after treatment with dexamethasone (data not shown), consistent with the here proposed non-genomic pathway.

Third, using pharmacological tools, we found that GR signaled to Mrp2 through c-Met, a receptor tyrosine kinase and its downstream effector pathway, MEK1/2, a protein ser/thr kinase. Thus rapid signaling by GR involved a downstream protein kinase cascade. Endogenously, this signaling cascade is triggered by HGF which activates c-Met by triggering receptor autophosphorylation. Although we do not show a direct generation of HGF upon dexamethasone treatment, we cannot explain our findings any differently than that the glucocorticoids signal through the growth factor. During development, HGF function is essential as knock-out mice for both ligand and receptor are embryonically lethal. HGF displays a unique feature in inducing "branching morphogenesis", a complex program of proliferation and ontogenesis in a number of different cell types, including renal cells (Davies and Fisher, 2002). Moreover, HGF is involved in tumor cell invasion both *in vivo* and *in vitro*. Perturbation of the HGF/c-Met axis leads to enhanced signaling that occurs in a wide range of human cancers, and elevated c-Met signals are characteristic of aggressive tumors with poor prognosis (Lai et al., 2009). Inhibition of the tyrosine kinase activity and blockade of the receptor/ligand interaction are strategies for novel targeted therapies in oncology (Eder et al., 2009). Inhibition of Mrp's by tyrosine kinase inhibitors may be a helpful mechanism in cancer therapy, because these efflux pumps notably contribute to development of drug resistance in chemotherapy (van de Water et al., 2005). On the other hand, chemicals that target the tyrosine kinase pathway, like tyrosine kinase inhibitors that block c-Met, may increase the sensitivity to xenobiotics and may also be the cause of reduced renal function associated with tyrosine kinase inhibitor treatment (Gafter-Gvili et al., 2009).

Non-genomic up-regulation of Mrp2 activity can result from insertion of the transporter into a region of the plasma membrane from which it can function, from covalent modification or from altered associations with membrane protein and lipids (Kubitz et al., 2005;Sabolic et al., 2002;Ito et al., 2005). In the present study, we found no changes in transporter protein expression in the luminal membrane of dexamethasone-treated tubules (immunostaining), nor any effect of the microtubule disruptor colchicine on dexamethasone stimulation of Mrp2 activity. These findings suggest that transporter activity increased through covalent modification or altered associations, within the membrane. Hegedus and co-workers (Hegedus et al., 2003) examined the effect of mutational changes at Ser1542 that mimic phosphorylation of the Mrp2 PDZ domain by PKC. They demonstrated that the phosphorylated form of MRP2 anchors PDZ

proteins stronger than the dephosphorylated form, suggesting a role in the distribution and routing of functional Mrp2. It is to be expected that c-Met influences Mrp2-mediated transport activity by another mechanism, because involvement of PKC was excluded in the present study and membrane insertion of endosomal vesicles expressing Mrp2 seems unlikely. Our experiments suggest that Met acts through the MEK1/2 signaling cascade, which in turn may phosphorylate and, thereby, activate ERK. More than 100 different proteins have been identified as substrates for the ERK1/2 signaling pathway (Ramos, 2008); thus, ERK activation affects an array of cellular functions. We used the sequences that were aligned in the paper by Long et al. (2011) and found 14 consensus Erk1/2 phosphorylation sites on zebrafish Mrp2. Of these, 7 are conserved when comparing the human and zebrafishfish transporter, indicating that Mrp2 is a potential target protein of Erk1/2. We speculate that the increase in Mrp2 function caused by glucocorticoids and activating MEK1/2 and ERK1/2 signaling is an essential way by which renal cells cope with accumulating waste products of metabolism during stress.

In conclusion, our results suggest a novel mechanism of rapid non-genomic induction of Mrp2-mediated transport in renal proximal tubules; no change in transporter protein expression was observed. Signaling involved glucocorticoids acting though the GR and the receptor tyrosine kinase c-Met. Downstream c-Met activates MEK1/2 finally resulting in increased Mrp2 function. In renal proximal tubules, Mrp2 pumps drugs and toxic substances into the urinary space. A better, more complete mapping of the regulatory pathways that alter Mrp2 activity may reveal new therapeutic and renoprotective targets.

Authorship Contributions

Participated in research design: DSM, FMW, KEW, FGMR, GF and RM

Conducted experiments: BP, KEW, FMW and RM

Performed data analysis: BP, DSM, FMW and RM

Wrote or contributed to the writing of the manuscript: BP, DSM, GF and RM

References

Bartholome B, Spies CM, Gaber T, Schuchmann S, Berki T, Kunkel D, Bienert M, Radbruch A, Burmester GR, Lauster R, Scheffold A and Buttgereit F (2004) Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. *Faseb J* **18**:70-80.

Bauer B, Hartz AM, Lucking JR, Yang X, Pollack GM and Miller DS (2008) Coordinated nuclear receptor regulation of the efflux transporter, Mrp2, and the phase-II metabolizing enzyme, GSTpi, at the blood-brain barrier. *J Cereb Blood Flow Metab* **28**:1222-1234.

Beck IM, Vanden BW, Vermeulen L, Yamamoto KR, Haegeman G and De BK (2009) Crosstalk in inflammation: the interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. *Endocr Rev* **30**:830-882.

Borski RJ (2000) Nongenomic membrane actions of glucocorticoids in vertebrates. *Trends Endocrinol Metab* **11**:427-436.

Breen CM, Sykes DB, Baehr C, Fricker G and Miller DS (2004) Fluorescein-methotrexate transport in rat choroid plexus analyzed using confocal microscopy. *Am J Physiol Renal Physiol* **287**:F562-F569.

Bury NR, Sturm A, Le Rouzic P, Lethimonier C, Ducouret B, Guiguen Y, Robinson-Rechavi M, Laudet V, Rafestin-Oblin ME and Prunet P (2003) Evidence for two distinct functional glucocorticoid receptors in teleost fish. *J Mol Endocrinol* **31**:141-156.

Cheng X and Klaassen CD (2006) Regulation of mRNA expression of xenobiotic transporters by the pregnane x receptor in mouse liver, kidney, and intestine. *Drug Metab Dispos* **34**:1863-1867.

Christensen JG, Schreck R, Burrows J, Kuruganti P, Chan E, Le P, Chen J, Wang X, Ruslim L, Blake R, Lipson KE, Ramphal J, Do S, Cui JJ, Cherrington JM and Mendel DB (2003) A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes in vitro and exhibits cytoreductive antitumor activity in vivo. *Cancer Res* **63**:7345-7355.

Dagan A, Gattineni J, Cook V and Baum M (2007) Prenatal programming of rat proximal tubule Na+/H+ exchanger by dexamethasone. *Am J Physiol Regul Integr Comp Physiol* **292**:R1230-R1235.

Davies JA and Fisher CE (2002) Genes and proteins in renal development. *Exp Nephrol* **10**:102-113.

de Vries WB, van den BP, Goldschmeding R, de Weger RA, Bal MP, van BF and van Oosterhout MF (2010) Neonatal dexamethasone treatment in the rat leads to kidney damage in adulthood. *Pediatr Res* **67**:72-76.

Eder JP, Vande Woude GF, Boerner SA and LoRusso PM (2009) Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. *Clin Cancer Res* **15**:2207-2214.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA and Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* **273**:18623-18632.

Gafter-Gvili A, Ram R, Gafter U, Shpilberg O and Raanani P (2009) Renal failure associated with tyrosine kinase inhibitors-Case report and review of the literature. *Leuk Res* **34**:123-127.

Gametchu B, Chen F, Sackey F, Powell C and Watson CS (1999) Plasma membrane-resident glucocorticoid receptors in rodent lymphoma and human leukemia models. *Steroids* **64**:107-119.

Heemskerk S, van KA, van den BL, Poelen GJ, Wouterse AC, Dijkman HB, Russel FG and Masereeuw R (2007) Nitric oxide differentially regulates renal ATP-binding cassette transporters during endotoxemia. *Pflugers Arch* **454**:321-334.

Hegedus T, Sessler T, Scott R, Thelin W, Bakos E, Varadi A, Szabo K, Homolya L, Milgram SL and Sarkadi B (2003) C-terminal phosphorylation of MRP2 modulates its interaction with PDZ proteins. *Biochem Biophys Res Commun* **302**:454-461.

Hulter HN, Licht JH, Bonner EL, Jr., Glynn RD and Sebastian A (1980) Effects of glucocorticoid steroids on renal and systemic acid-base metabolism. *Am J Physiol* **239**:F30-F43.

Ito K, Wakabayashi T and Horie T (2005) Mrp2/Abcc2 transport activity is stimulated by protein kinase Calpha in a baculo virus co-expression system. *Life Sci* **77**:539-550.

Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM and Edwards PA (2002) Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* **277**:2908-2915.

Kubitz R, Helmer A and Haussinger D (2005) Biliary transport systems: short-term regulation. *Methods Enzymol* **400**:542-557.

Kumar S, Allen DA, Kieswich JE, Patel NS, Harwood S, Mazzon E, Cuzzocrea S, Raftery MJ, Thiemermann C and Yaqoob MM (2009) Dexamethasone Ameliorates Renal Ischemia-Reperfusion Injury. *J Am Soc Nephrol* **20**:2412-2425.

Lai AZ, Abella JV and Park M (2009) Crosstalk in Met receptor oncogenesis. *Trends Cell Biol* **19**:542-551.

Long Y, Li Q, Zhong S, Wang Y and Cui Z (2011) Molecular characterization and functions of zebrafish ABCC2 in cellular efflux of heavy metals. *Comp Biochem Physiol C Toxicol Pharmacol* **153**:381-391.

Lowenberg M, Stahn C, Hommes DW and Buttgereit F (2008) Novel insights into mechanisms of glucocorticoid action and the development of new glucocorticoid receptor ligands. *Steroids* **73**:1025-1029.

Masereeuw R, Russel FG and Miller DS (1996) Multiple pathways of organic anion secretion in renal proximal tubule revealed by confocal microscopy. *Am J Physiol* **271**:F1173-F1182.

Masereeuw R, Terlouw SA, van Aubel RA, Russel FG and Miller DS (2000) Endothelin B receptor-mediated regulation of ATP-driven drug secretion in renal proximal tubule. *Mol Pharmacol* **57**:59-67.

Miller DS, Letcher S and Barnes DM (1996) Fluorescence imaging study of organic anion transport from renal proximal tubule cell to lumen. *Am J Physiol* **271**:F508-F520.

Miller DS and Pritchard JB (1997) Dual pathways for organic anion secretion in renal proximal tubule. *J Exp Zool* **279**:462-470.

Moore LB, Maglich JM, McKee DD, Wisely B, Willson TM, Kliewer SA, Lambert MH and Moore JT (2002) Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Mol Endocrinol* **16**:977-986.

Notenboom S, Miller DS, Kuik LH, Smits P, Russel FG and Masereeuw R (2005) Short-term exposure of renal proximal tubules to gentamicin increases long-term multidrug resistance protein 2 (Abcc2) transport function and reduces nephrotoxicant sensitivity. *J Pharmacol Exp Ther* **315**:912-920.

Notenboom S, Miller DS, Smits P, Russel FG and Masereeuw R (2004) Involvement of guanylyl cyclase and cGMP in the regulation of Mrp2-mediated transport in the proximal tubule. *Am J Physiol Renal Physiol* **287**:F33-F38.

Notenboom S, Wouterse AC, Peters B, Kuik LH, Heemskerk S, Russel FG and Masereeuw R (2006) Increased apical insertion of the multidrug resistance protein 2 (MRP2/ABCC2) in renal proximal tubules following gentamicin exposure. *J Pharmacol Exp Ther* **318**:1194-1202.

Orchinik M, Moore FL and Rose JD (1994) Mechanistic and functional studies of rapid corticosteroid actions. *Ann N Y Acad Sci* **746**:101-112.

Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD and Lehmann JM (1999) Bile acids: natural ligands for an orphan nuclear receptor. *Science* **284**:1365-1368.

Ramos JW (2008) The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. *Int J Biochem Cell Biol* **40**:2707-2719.

Renfro MW, King GB and Laurendeau NM (1999) Quantitative hydroxyl concentration time-series measurements in turbulent nonpremixed flames. *Appl Opt* **38**:4596-4608.

Roy B and Rai U (2009) Genomic and non-genomic effect of cortisol on phagocytosis in freshwater teleost Channa punctatus: an in vitro study. *Steroids* **74**:449-455.

Sabolic I, Herak-Kramberger CM, Ljubojevic M, Biemesderfer D and Brown D (2002) NHE3 and NHERF are targeted to the basolateral membrane in proximal tubules of colchicine-treated rats. *Kidney Int* **61**:1351-1364.

Saklatvala J, Rawlinson L, Waller RJ, Sarsfield S, Lee JC, Morton LF, Barnes MJ and Farndale RW (1996) Role for p38 mitogen-activated protein kinase in platelet aggregation caused by collagen or a thromboxane analogue. *J Biol Chem* **271**:6586-6589.

Schramm U, Fricker G, Wenger R and Miller DS (1995) P-glycoprotein-mediated secretion of a fluorescent cyclosporin analogue by teleost renal proximal tubules. *Am J Physiol* **268**:F46-F52.

Tapley P, Lamballe F and Barbacid M (1992) K252a is a selective inhibitor of the tyrosine protein kinase activity of the trk family of oncogenes and neurotrophin receptors. *Oncogene* **7**:371-381.

Teitsma CA, Anglade I, Toutirais G, Munoz-Cueto JA, Saligaut D, Ducouret B and Kah O (1998) Immunohistochemical localization of glucocorticoid receptors in the forebrain of the rainbow trout (Oncorhynchus mykiss). *J Comp Neurol* **401**:395-410.

Terlouw SA, Graeff C, Smeets PH, Fricker G, Russel FG, Masereeuw R and Miller DS (2002) Short- and long-term influences of heavy metals on anionic drug efflux from renal proximal tubule. *J Pharmacol Exp Ther* **301**:578-585.

Terlouw SA, Masereeuw R, Russel FG and Miller DS (2001) Nephrotoxicants induce endothelin release and signaling in renal proximal tubules: effect on drug efflux. *Mol Pharmacol* **59**:1433-1440.

van de Water FM, Masereeuw R and Russel FG (2005) Function and regulation of multidrug resistance proteins (MRPs) in the renal elimination of organic anions. *Drug Metab Rev* **37**:457-485.

Vargas GA, Hoeflich A and Jehle PM (2000) Hepatocyte growth factor in renal failure: promise and reality. *Kidney Int* **57**:1426-1436.

Vlahos CJ, Matter WF, Hui KY and Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* **269**:5241-5248.

Wang D, Zhang H, Lang F and Yun CC (2007) Acute activation of NHE3 by dexamethasone correlates with activation of SGK1 and requires a functional glucocorticoid receptor. *Am J Physiol Cell Physiol* **292**:C396-C404.

Zhang H, LeCulyse E, Liu L, Hu M, Matoney L, Zhu W and Yan B (1999) Rat pregnane X receptor: molecular cloning, tissue distribution, and xenobiotic regulation. *Arch Biochem Biophys* **368**:14-22.

Footnotes

Legends for figures

Figure 1. A-D; Representative images of renal tubules incubated with or without (control, A) 1 µM dexamethasone (B), cortisol (C; 1 µM) or the inactive analog corticosterone (D; 1 µM). E; Stimulation by dexamethasone is highest at 1µM. Tubules were incubated with or without (control) 0.25-10µM dexamethasone for 3 h. F; Rapid stimulation of FL-MTX transport by dexamethasone. Tubules were incubated with FL-MTX until steady state after which 1µM dexamethasone was added. The fluorescence intensities in lumen and cell compartments are depicted as percentage of the fluorescence in control lumen. Mean values ± S.E.M. are shown for 48-231(E) and 15-48 (F) tubules. Significantly different from control: **$p<0.01$, ***$p<0.001$.

Figure 2. Glucocorticoids induce Mrp2-mediated transport via the GR and not via the MR. Tubules were incubated with or without (control) 1 µM dexamethasone (dex; A), cortisol (B; 1 µM) or triamcinolone acetonide (TA; C; 1 µM), 0.5 µM RU486 or the combination of glucocorticoids and RU486. Lack of involvement of the MR is supported by an absence of effective reversal of dexamethasone-mediated induction by 0.5 µM spironolactone (D). The fluorescence intensities of lumen and cell compartments are depicted as percentage of the fluorescence in control lumen. Mean values ± S.E.M. are shown for 11-16 (A), 9-11 (B), 16-23 (C) and 22-42 (D) tubules. Significantly different from control: *$p<0.05$, **$p<0.01$, ***$p<0.001$, or dexamethasone treatment, [#] $p<0.05$; [##] $p<0.01$; [###] $p<0.001$.

Figure 3. Dexamethasone stimulates Mrp2 non-genomically without an increase in expression in killifish proximal tubules. A; Tubules were incubated with 1 µM cortisone (control condition), 1 µM dexamethasone (dex), 100 µg/mL cycloheximide (cyclo) or both. B; Tubules were incubated without (control) or with 1 µM dexamethasone (dex) and/or actinomycin-D (AcD; 1 µM). The fluorescence intensities in lumen and cell compartments are depicted as a percentage of the fluorescence from control. Mean values ± S.E.M. are shown for 10-16 (A) and 13-17 (B) tubules. C-F; Representative images of Mrp2 immunostaining after 1 hour exposure with or whithout (control; C + E) 1 µM dexamethasone (dex; D + F), as described in concise methods. G; Mean fluorescence intensities of Mrp2 immunostaining after 1 hour exposure with or whithout 1 µM dex measured for 15 tubules ± S.E.M. are shown. H; Tubules were incubated without

(control) or with 1 µM dexamethasone (dex) and/or colchicine (colch; 50 µM). Mean values ± S.E.M. are shown for 15-30 tubules. Significantly different from control treatment: *p<0.05, **p<0.01, ***p<0.001. Significantly different from dexamethasone: #p<0.05, ##p<0.01, ###p<0.001.

Figure 4. The iNOS/PKC signaling pathway is not activated by dexamethasone. A. Tubules were incubated for 3 h with or without (control) 1µM dexamethasone (dex), 50 µM NG-methyl-L-arginine (L-NMMA) or both. B. Tubules were incubated for 1 h with or without (control) 1 µM dexamethasone (dex), 100 nM bisindolylmaleimide (BIM) or both. The fluorescence intensities in lumen and cell compartments are depicted as a percentage of fluorescence in control lumen. Mean values ±S.E.M. are shown for 26-231 (A) and 11-152 (B) tubules. Significantly different from control: *p<0.05, ***p<0.001. Significantly different from dexamethasone: ###p<0.001.

Figure 5. Induction of Mrp2 activity is regulated by tyrosine kinases, and more specific by c-Met. Tubules were incubated without (control) or with 1 µM dexamethasone (dex), 10 µM genistein (gen; A), 1 µM K252a (B), 1 µM PHA-665752 (PHA; C) or the combination of dexamethasone and the kinase inhibitors. The fluorescence intensities in lumen and cell compartments are depicted as a percentage of the fluorescence in control lumen. Mean values ± S.E.M. are shown for 9-11 (A), 8-18 (B) and 10-17 (C) tubules. Significantly different from control: **p<0.01, ***p<0.001. Significantly different from dexamethasone: #p<0.05, ###p<0.001.

Figure 6. HGF stimulates Mrp2-mediated transport rapidly through c-Met activation, but not involving GR signaling. A; Stimulation by HGF is highest at 25 nM. Tubules were incubated with or without (control) HGF for 1 h. B; Rapid stimulation of FL-MTX transport by HGF. Tubules were incubated with FL-MTX for 30 min until steady state after which 25 nM HGF was added. C; Lack of involvement of the GR is supported by an absence of effective reversal of HGF-mediated induction by 0.5 µM RU486. D; HGF-induced increase in FL-MTX secretion is reversed by co-treatment with 1 µM PHA-665752. The fluorescence intensities in lumen and cell compartments are depicted as percentage of the fluorescence in control lumen. Mean values ± S.E.M. are shown for 11-18 (A), 12-30 (B), 10-18 (C) and 15-47 (D) tubules.

Significantly different from control: *p<0.05, **p<0.01, ***p<0.001. Significantly different from HGF: ##p<0.01, ### p<0.001.

Figure 7. Dexamethasone and HGF stimulate Mrp2 through MEK1/2 signaling. A; Tubules were incubated without (control) or with 1 μM dexamethasone (dex) or 25 nM HGF and the MEK1/2 inhibitor U0126 (10 μM) for 1 h. B; Co-treatment with the PI3K inhibitor LY294002 (10 μM) appears to be ineffective in reversal of the dex or HGF induced increase in FL-MTX secretion. C; Also up to 10 μM of SB203580 (SB) was not efficient in abolishing the dexamethasone-induced effect, indicating absence of p-Akt signaling. The fluorescence intensities in lumen and cell compartments are depicted as percentage of the fluorescence in control lumen. Mean values ± S.E.M. are shown for 20-39 (A), 10-19 (B) and 11-21 (C) tubules. Significantly different from control: *p<0.05, ***p<0.001. Significantly different from dexamethasone or HGF: ### p<0.001.
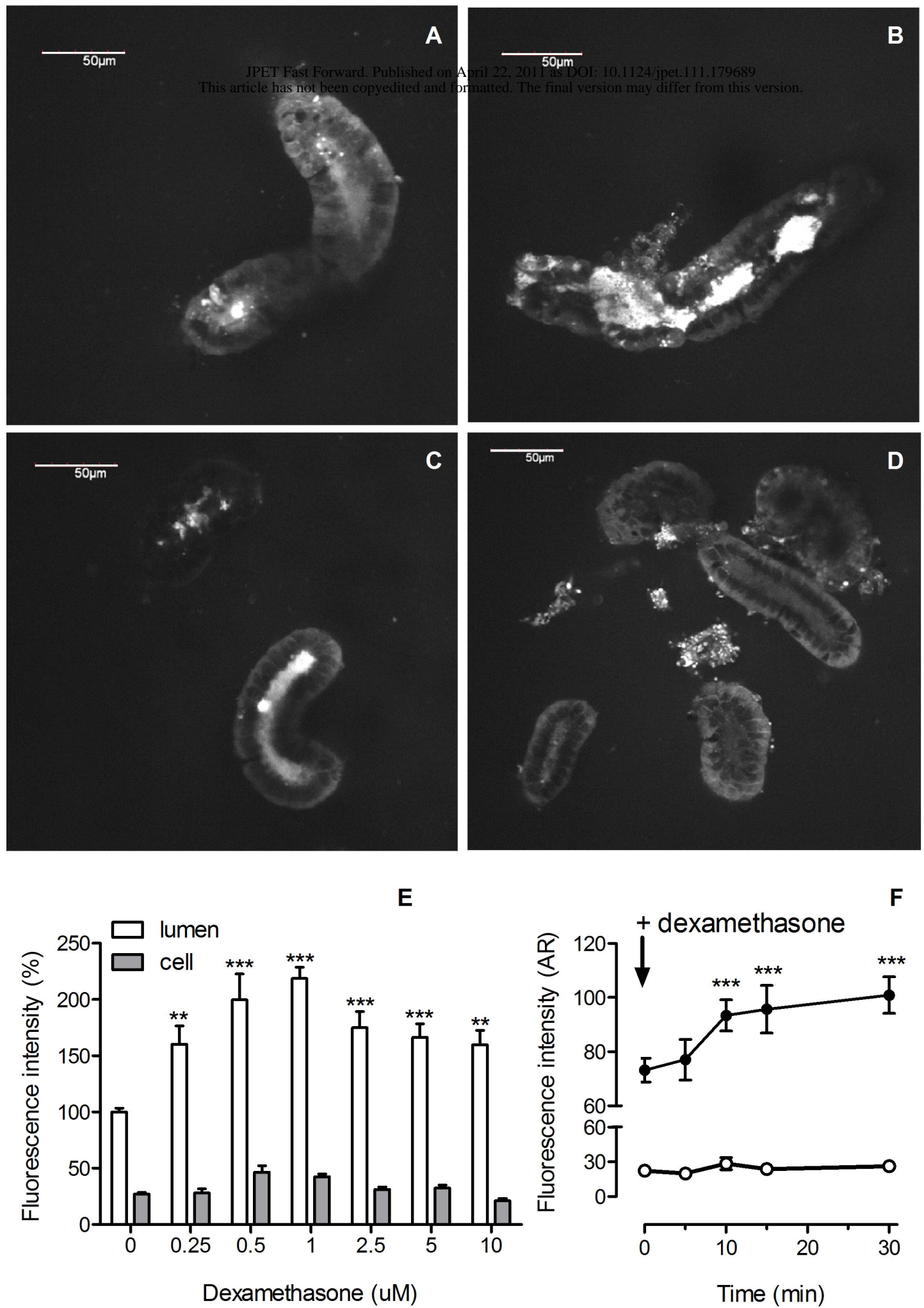
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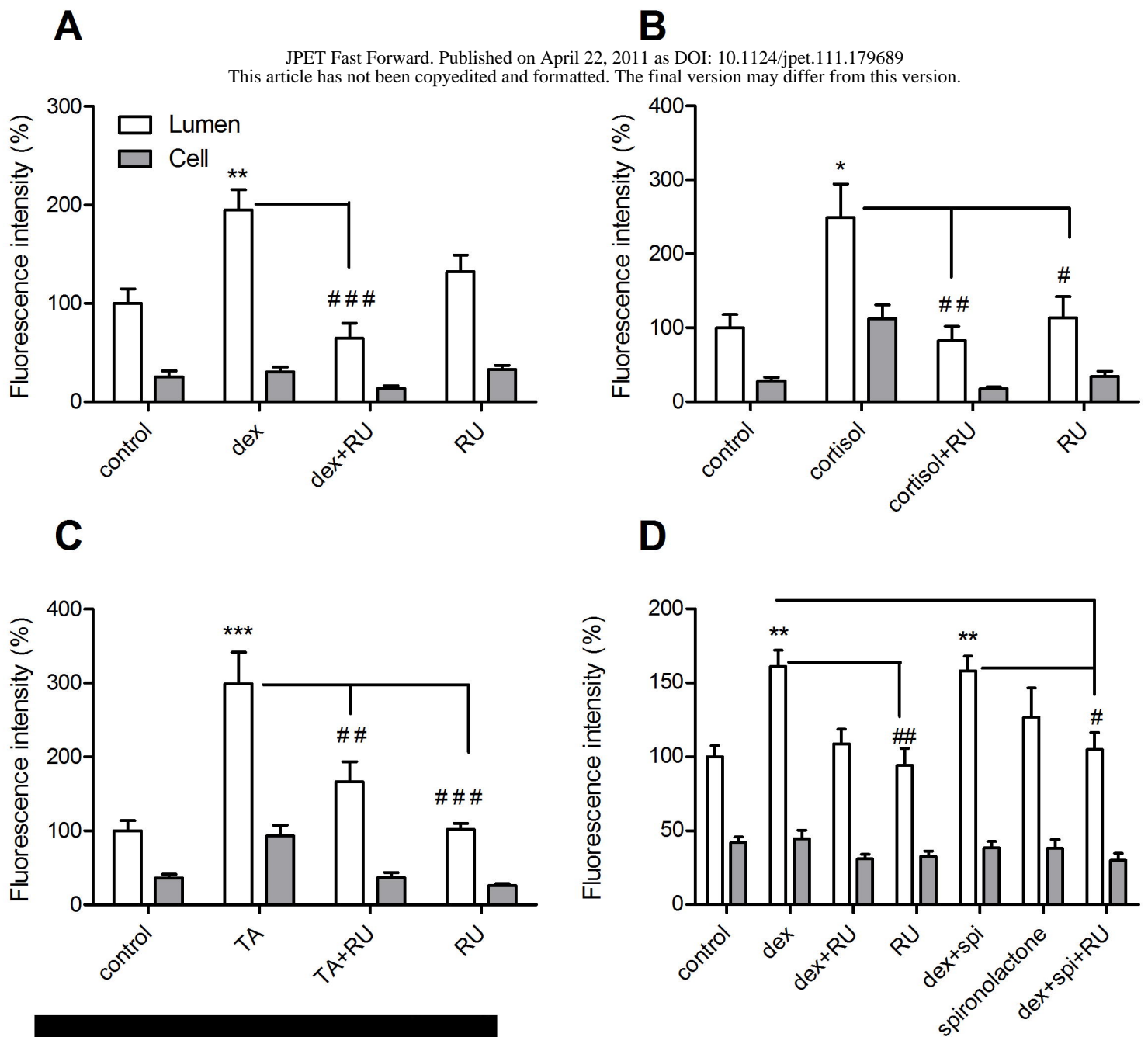
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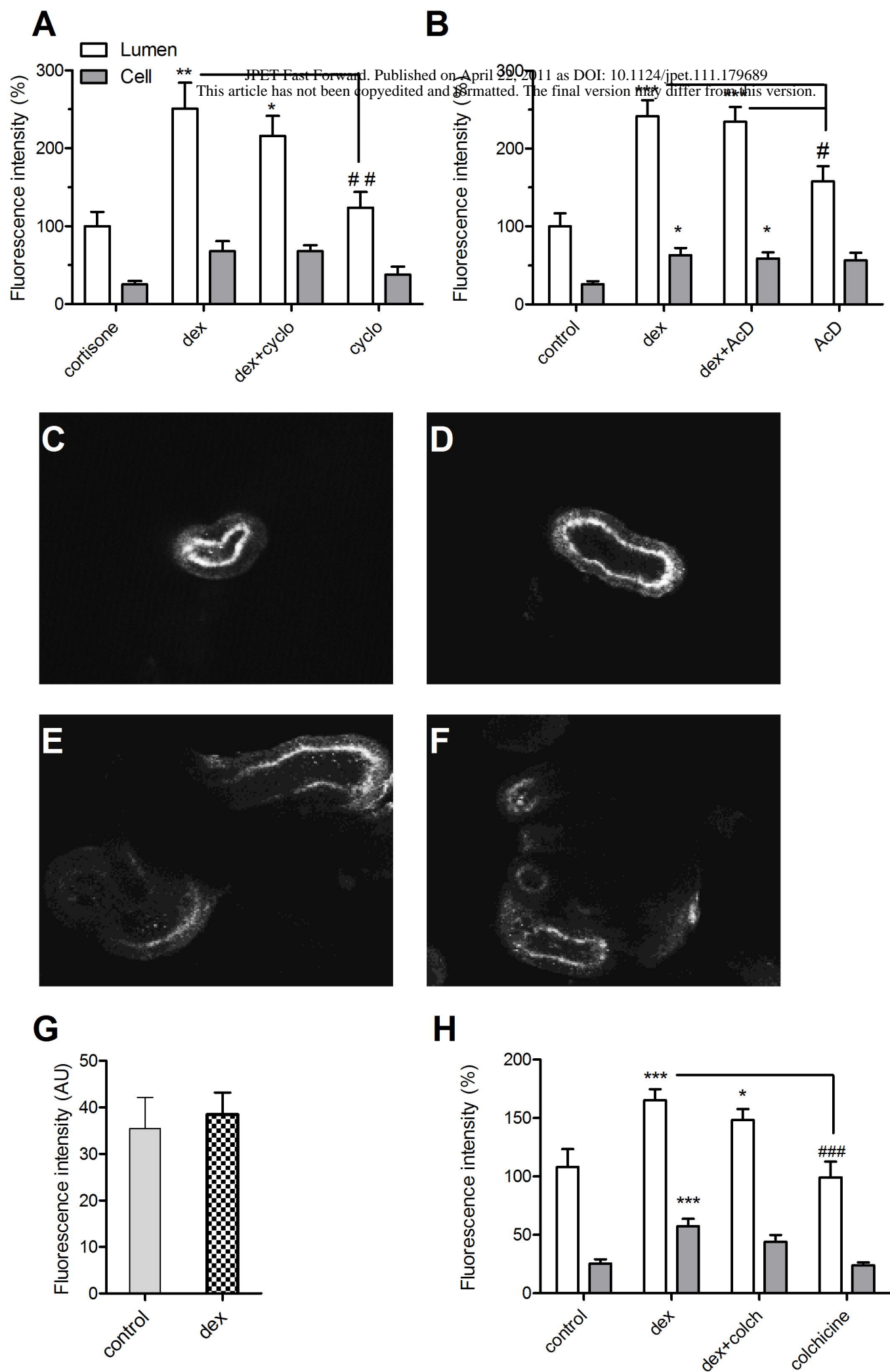
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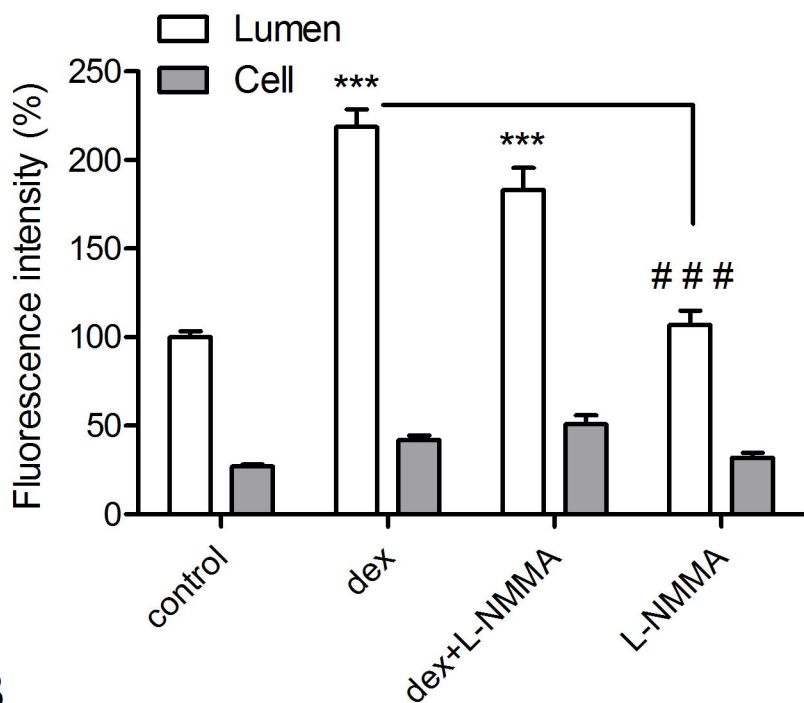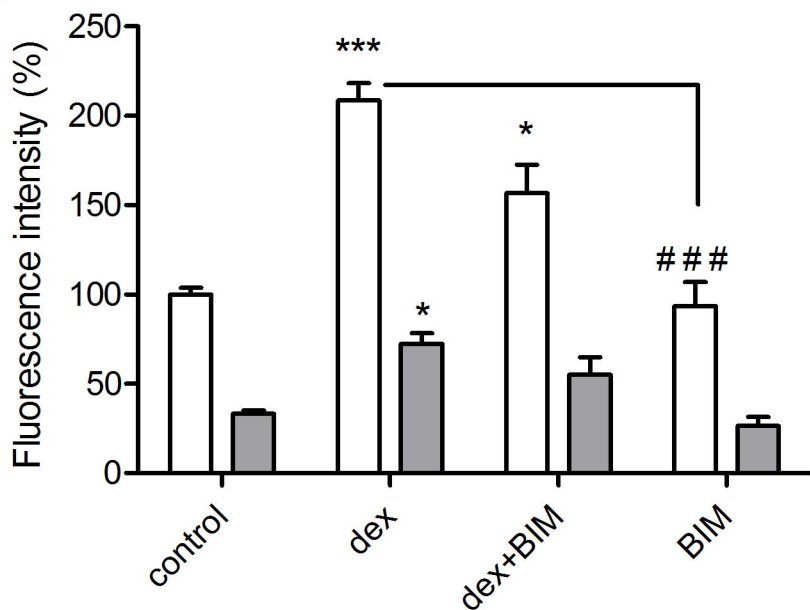
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