Biphasic modulation of cAMP levels by the contraceptive nomegestrol acetate. Impact on P-glycoprotein expression and activity in hepatic cells.

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Abstract

ABC transporters are key players in drug excretion with alterations in their expression and activity by therapeutic agents potentially leading to drug-drug interactions. The interaction potential of nomegestrol acetate (NMGA), a synthetic progestogen increasingly used as oral contraceptive, had never been explored. In this work we evaluated (1) the effect of NMGA on ABC transporters in the human hepatic cell line HepG2 and (2) the underlying molecular mechanism. NMGA (5, 50 and 500 nM) increased P-glycoprotein (P-gp) expression at both protein and mRNA levels and reduced intracellular calcein accumulation, indicating an increase also in transporter activity. This up-regulation of P-gp was corroborated in Huh7 cells and was independent of the classical progesterone receptor. Instead, using a siRNA-mediated silencing approach, we demonstrated the involvement of membrane progesterone receptor α. Moreover, we found that the activation of this receptor by NMGA led to a falling-rising profile in intracellular cAMP levels and protein kinase A activity over time, ultimately leading to transcriptional P-gp up-regulation. Finally, we identified inhibitory G protein and phosphodiesterases as mediators of this novel biphasic modulation. These results demonstrate the ability of NMGA to selectively up-regulate hepatic P-gp expression and activity and constitute the first report of ABC transporter modulation by membrane progesterone receptor α. If a similar regulation took place in vivo, decreased bioavailability and therapeutic efficacy of NMGA-coadministered P-gp substrates could be expected. This holds special importance considering long-term administration of NMGA and broad substrate specificity of P-gp.

Keywords

ABC transporters; nomegestrol acetate; cAMP; membrane progesterone receptor; drug-drug interactions.

1. Introduction

Drug transporters belonging to the ATP binding cassette (ABC) superfamily play a key role in the disposition of therapeutic agents. Among them, the P-glycoprotein (P-gp/*ABCB1*), the multidrug resistance-associated proteins 2 and 3 (MRP2/*ABCC2* and MRP3/*ABCC3*) and the breast cancer resistance protein (BCRP/*ABCG2*) transport a wide range of prescription drugs and their metabolites. In the liver, they localize to the canalicular membrane (P-gp, MRP2 and BCRP) or the basolateral membrane (MRP3) of the hepatocyte and actively extrude their substrates into the bile or into the liver sinusoids, respectively, thus affecting their pharmacokinetics [1]. Modulation of ABC transporters by drugs represents a common issue potentially resulting in drug-drug interactions. Increasingly preclinical and clinical evidence associates alterations in transporter expression with a modified clearance of coadministered drugs and thus with changes in their therapeutic efficacy and toxicity. As a well-studied example, hyperforin, a major active principle of the natural antidepressant St. John's wort, induces P-gp expression and activity [2]. In line with this observation, a reduced bioavailability of the P-gp substrate digoxin in healthy volunteers receiving hyperforin has been described [3]. Similarly, modulation of MRP2 may also lead to unwanted drug-drug interactions. For instance, rifampicin has been described to induce expression of MRP2 *in vivo* [4] and, this way, to inhibit the analgesic effect of morphine [5]. Other drug-drug interactions have been reported, for instance, for the MRP3 substrate acetaminophen-glucuronide [6] and the BCRP substrate methotrexate [7]. In general, most of the reported cases of ABC transporter regulation by drugs can be explained in terms of activation of nuclear receptors or transcription factors as well as post-transcriptional mechanisms finally impacting in the expression levels of the different transporters [8, 9].

Nomegestrol acetate (NMGA) is a potent synthetic progestogen originally used alone for treatment of uterine disorders or in combination with estradiol (E2) as hormonal replacement therapy in postmenopausal women. During the last years, a combination of NMGA and E2 has been approved in many countries for its use in oral contraception. NMGA is characterized by binding with high affinity to the progesterone receptor (PR) and by exhibiting high antigonadotropic activity [10, 11]. Unlike other progestogens used in oral contraception, NMGA does not exhibit androgenic effects and, in combination with E2, it displays fewer adverse effects than other combined oral contraceptives [12, 13]. Thus, NMGA-E2 formulations emerge as a promising high efficacy-few side effect combination that may gain even more acceptance among women and physicians in the future. Noteworthy, due to its relatively recent launch into the market, there is scarce information on the potential of NMGA to modulate ABC transporters and thus to mediate drug-drug interactions. This bears particular importance considering that contraceptive therapies are usually taken during a long part of the women's reproductive age [14], during which other drugs are very likely to be coadministered.

Progestogens mediate their physiological and pharmacological effects through binding to different kind of receptors. The classical progesterone receptors (PR A/B) are nuclear receptors exerting their effect by binding to response elements in the promoter of target genes [15]. The progesterone receptor membrane component 1 (PGRMC) is part of a multi-protein progesterone-binding complex already related to different physiological and pathological processes [16]. Furthermore, the membrane progesterone receptors (mPR) are capable of activating inhibitory G protein (G_i) mediating both genomic and non-genomic effects [17]. The main mPR isoforms present in human liver are mPRα (*PAQR7*) and mPRγ (*PAQR5*), whereby *PAQR7* expression was reported to be at least 4 orders of magnitude higher than *PAQR5*

[18]. Modulation of ABC transporters by PR has been already described [19]. Similarly, modulation by fluctuation of cAMP levels, one of the major downstream processes that could be involved after mPR activation, has been reported for P-gp [20], MRP2 [21] and MRP3 [22]. However, a potential association between this receptor and transporter regulation has not been investigated before. Since the activation of the above-mentioned receptors by NMGA was either demonstrated or seems feasible, a modulation of ABC transporters by this progestogen could be expected. The aim of the current work was to assess the effect of NMGA on the expression and activity of pharmacologically relevant ABC transporters and characterize the underlying molecular mechanisms.

Our data indicates a selective up-regulation of P-gp expression and activity by NMGA in human hepatic cell lines. Moreover, our observations highlight the involvement of the mPRα in the regulatory mechanism, representing the first study describing a modulation of an ABC protein by an mPR. Furthermore, our results point to a biphasic falling-rising profile in the cAMP levels as a major mechanism responsible for P-gp up-regulation.

2. Materials and methods

2.1. Chemicals

AG-205, nomegestrol acetate (NMGA), cell culture media and all supplements were from Sigma-Aldrich (St. Louis, USA). Ponceau S, ethanol, aprotinin, leupeptin, pepstatin and pefabloc were from Carl Roth (Karlsruhe, Germany). 3-isobutyl-1-methylxanthine (IBMX), actinomycin D, calcein-AM, H-89 dihydrochloride (H89), KT5720, Rp-8-Br-cAMP, mifepristone (RU 486), PSC833, Ro 20-1724 and SC68376 were from Santa Cruz Biotechnology (Heidelberg, Germany). *Bordetella pertussis* toxin (PTX) was from Enzo Life Sciences (Farmingdale, USA). PD98059 was from Calbiochem (San Diego, USA). Polyvinylidene difluoride (PVDF) membranes were from GE Healthcare (Little Chalfont, UK). DharmaFECT4 Transfection Reagent was from Dharmacon (Lafayette, USA). DMSO was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade purity.

2.2. Cell culture

HepG2 cells were purchased from the American Tissue Culture Collection (Rockville, USA) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Huh7 cells were a kind gift from Prof. Dr. Ralf Bartenschlager (Heidelberg University Hospital, Germany) and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL. Both cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 [23]. All experiments were performed with cells differing in not more than 10 passages.

For the analysis of ABC transporter and mPR protein expression, P-gp, protein kinase A (PKA) and phosphodiesterase (PDE) activity studies, and intracellular cAMP determinations, cells were seeded in 6-well plates at a density of $5.0x10^5$ cells/well. For mRNA expression studies, cells were seeded in T25 flasks at a density of $1.0x10^6$ cells/flask and cultured for 24 h. For siRNA-mediated mPRα knockdown, cells were seeded in 12-well plates at a density of $5.0x10^4$ cells/well. In all cases, cells were cultured for 24 h after seeding and then incubated in treatment medium, consisting of growing culture medium and specific reagents. Particular treatment details are provided below in the corresponding subsection.

2.3. Western blot studies

We first evaluated the effect of NMGA on ABC transporter protein expression. NMGA was tested at different concentrations (0.5-500 nM) aimed to cover the reported range in plasma of individuals under NMGA treatment (5-40 nM) [13]. For this purpose, NMGA was dissolved in DMSO as a 1000x stock solution. Participation of PR A/B, PGRMC1 and Gi protein as mediators of NMGA effect on P-gp expression was explored using their inhibitors RU 486 (1 µM) [24], AG-205 (20 µM) [25] and PTX (200 ng/mL) [26], respectively. In order to assess the participation of PKA as mediator of NMGA effect, KT5720 (1 µM), H-89 (10 µM) [21] or Rp-8-Br-cAMPS (100 µM) [27] were added to the treatment medium. To evaluate the involvement of p38 and ERK1/2 MAP kinases SC68376 (10 µM) [28] and PD98059 (20 µM) [29] were used, respectively. RU 486 was dissolved in ethanol and added from a 10000X stock solution. AG-205, KT5720, H-89, SC68376 and PD98059 were dissolved in DMSO and added from 1000x stock solutions. PTX was reconstituted with sterile distilled water and added from a 500x stock solution. Rp-8-Br-cAMPS was

dissolved in sterile distilled water and added from a 100x stock solution. The corresponding vehicles were used in control incubations. The final concentration of DMSO and ethanol in culture media was always kept below 0.2% (V/V). In all cases, cells were exposed to treatment medium for 48 h with exception of PKA activity study, in which cells were treated with 50 nM NMGA for 6 and 22 h. At the end of incubations, cells were harvested and subjected to western blot analysis as previously described by Rigalli et al. [30]. Briefly, cells were rinsed with PBS and incubated for 30 min in RIPA buffer (Thermo Fisher Scientific, Waltham, USA) supplemented with aprotinin (1 µg/mL), leupeptin (5 µg/mL), pepstatin (1 µg/mL) and pefabloc (1 mg/mL) as protease inhibitors. Cell lysis was accomplished by passing through a 25G needle. Then, lysates were subjected to protein quantification using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA). Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane and incubated in blocking buffer (3% BSA in TBS with 0.3% Tween 20) for 1 h. Then, membranes were sequentially incubated with primary and secondary antibody solutions and developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, USA). For low abundance protein detection (mPRγ), the high-sensitive WesternBright Sirius Chemiluminescent Detection Kit (Advansta Inc., Menlo Park, USA) was used. Finally, optical density of the bands was quantified using ImageJ software (NIH, Bethesda, USA). For PKA activity study, a Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Danvers, USA) was also added to the RIPA buffer. For mPR detection, human liver homogenate (mixed gender, pool of 20 donors, catalog number H0610.H) was supplied by XenoTech (Kansas City, USA) and HepG2 lysates were prepared from untreated cells as described above. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Uniformity of loading and transfer was also checked through staining with

Ponceau S. Primary antibodies were: anti-P-gp: C219 (1:1000) from Calbiochem (San Diego, USA); anti-BCRP: BXP-21 (1:1000) and anti-GAPDH: G-9 (1:1000) from Santa Cruz Biotechnology (Heidelberg, Germany); anti-MRP2: M_2III-6 (1:1000) from Enzo Life Sciences (Farmingdale, USA); anti-MRP3: M_3II-21 (1:1000) from Sigma-Aldrich (Taufkirchen, Germany); anti-Phospho-(Ser/Thr) PKA substrate (9621) (1:1000) from Cell Signaling Technology (Danvers, USA); anti-mPRα (ab123979) (1:100) and anti-mPRγ (ab79517) (1:500) from Abcam (Cambridge, UK).

2.4. Real time RT-PCR studies

Cells were seeded as described in 2.2. *ABCB1* mRNA level was evaluated for those NMGA concentrations showing alterations in P-gp protein expression. For *ABCB1* mRNA stability study, cells were treated with NMGA (50 nM) or vehicle for 16 h and mRNA synthesis was inhibited by adding actinomycin D (5 µg/mL). Cells were harvested at 0, 2, 4 and 8 h after actinomycin D addition. For *PDE4D* mRNA expression, cells were treated with NMGA (50 nM, 8 h). After treatments, cells were rinsed with ice cold PBS and subjected to total RNA isolation using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, USA). cDNA was synthesized with the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA). The most suitable housekeeping gene was identified as described [30]. Among the tested housekeeping genes (*hypoxanthine-phosphoribosyltransferase 1, HPRT; ribosomal protein L13, RPL13; glucose-6-phosphate dehydrogenase, G6PDH; β-glucuronidase, GU; RNA polymerase II, RPII and 60S human acidic ribosomal protein P1, HUPO*), *HUPO* was the most stable under our experimental conditions. *PDE4D* primers were GGCAGGGTCAAACTGAGAAATT (forward) and TGACTGCCACTGTCCTTTTCC (reverse) and allow the detection of all the splicing variants of *PDE4D* gene [31].

PAQR7 (mPRα) primers were: CTGAAGTTTGCCTGACACCA (forward) and AATAGAAGCGCCAGGTCTGA (reverse). *PAQR5* (mPRγ) primers were: GGTGCCGTCAACCTCTTC (forward) and CATCCGGGAACGTGTATG (reverse). All other primers used in the current work were described in Theile et al. [32].

2.5. P-gp activity assay

P-gp activity was evaluated assessing the intracellular amount of the fluorescent compound calcein, which is intracellularly generated by hydrolysis of the P-gp substrate calcein-AM and inversely correlates with transporter activity [23, 33]. Cells were seeded as described in 2.2. and treated with 5-500 nM NMGA for 48 h. After treatment, cells were trypsinized, resuspended and incubated in growth medium containing the membrane permeable precursor calcein-AM (0.5 µM, 15 min). Then, cells were rinsed and resuspended in cold PBS. Calcein accumulation was quantified by flow cytometry using a Cell Sorter BD FACSAria II device (BD Biosciences, San Jose, USA) with a blue laser (488 nm, 20 mW) and FITC detection filter (530/30 nm). P-gp participation in the modulation of calcein accumulation was verified using the selective inhibitor PSC833 (10 µM) [33].

2.6. siRNA-mediated mPRα knockdown

Transfection conditions regarding initial cell density, transfection reagent volume and siRNA concentration were optimized prior to experiments. Cells were seeded as described in section 2.2. Transfection media consisting of mixtures of mPRα siRNA (ON-TARGETplus SMARTpool siRNA, L-008033-00, Dharmacon, Lafayette, USA) or non-targeting control siRNA (Control siRNA-A, Santa Cruz Biotechnology, Heidelberg, Germany) with DharmaFECT4 Transfection Reagent (4 µL/well) were

prepared according to manufacturer's instructions to reach a final siRNA concentration of 50 nM. Cells were incubated in the corresponding transfection medium for 24 h. Then, transfection media were replaced by treatment medium containing NMGA (5-500 nM). Cells were further incubated for 48 h and then subjected to western blot analysis for mPRα, P-gp and GAPDH as described in section 2.3.

2.7. Intracellular cAMP determination

To evaluate the time course of intracellular cAMP levels in response to NMGA, cells were seeded as described in section 2.2 and incubated with treatment medium containing NMGA (50 nM) for 15 min, 10, 14, 18 and 24 h. To evaluate the participation of PDEs in the increase in cAMP levels after 14 h of treatment, we repeated the experiment at that time point in the presence of IBMX (500 µM, non-selective PDE inhibitor) and Ro 20-1724 (100 µM, PDE4 selective inhibitor) [34]. Then, treatment medium was removed, cells were washed with PBS and lysed in HCl (0.1 M, 20 min) at room temperature to stop PDEs activity and stabilize the released cAMP [35]. Afterwards, cAMP levels were determined using cAMP Select ELISA kit (Cayman Chemical, Ann Arbor, USA) according to the manufacturer's instructions.

2.8. PDE activity

To confirm PDE involvement in late intracellular cAMP increase, cells seeded as described in 2.2. were treated with 50 nM NMGA for 11 h. They were then rinsed with cold Tris buffer and lysed using NP-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris-HCl, pH=7.4) supplemented with aprotinin (1 µg/mL), leupeptin (5 µg/mL), pepstatin (1 µg/mL) and pefabloc (1 mg/mL) as protease inhibitors. PDE activity was measured in cell lysates using a colorimetric PDE Activity Assay Kit

(Abcam, Cambridge, UK) based on the sequential hydrolysis of cyclic nucleotides by PDE and 5' nucleotidase. The released phosphate by enzymatic cleavage is directly proportional to PDE activity and quantified using a modified Malachite Green reagent.

2.9. Statistical analysis

All results are expressed as mean ± standard deviation. All experiments were performed at least in triplicates. Statistical comparisons were performed through the Student's t-test or one-way ANOVA followed by Tukey post-hoc test, for 2 or more experimental groups, respectively. Statistical significance was set at $p < 0.05$. Statistical analysis and artwork were performed using Graph Pad Prism 7.0d (Graph Pad Software, La Jolla, USA).

3. Results

3.1. NMGA increases P-gp expression and activity in hepatic cell lines

We first evaluated the effect of different concentrations of NMGA on ABC transporter protein expression by western blot. HepG2 cells treated with 5, 50 or 500 nM NMGA for 48 h showed a significant, concentration-independent increase in P-gp expression (+83%, +77% and +93%, respectively) with respect to control cells (Fig 1a). The inducing effect was not observed for the lowest concentration tested (0.5 nM). The inducing effect on P-gp was confirmed in the Huh7 cell line, as treatment with NMGA 5, 50 and 500 nM resulted in increased protein expression (+49%, +46% and +67%, respectively) with respect to control cells. As in HepG2 cells, NMGA 0.5 nM failed to increase P-gp expression (Fig 1b). BCRP, MRP2 and MRP3 protein expression was not affected by NMGA treatments (Fig 2).

We further evaluated the molecular mechanisms and the pharmacological relevance of P-gp induction using HepG2 cells as a representative model. First, we quantified P-gp expression at the mRNA level by real time RT-PCR. In line with the previous result, an increase in *ABCB1* mRNA level after 24 h treatment with 5, 50 and 500 nM NMGA was observed (Fig 1c). *ABCB1* mRNA stability was not affected (data not shown), suggesting a transcriptional effect of NMGA.

To characterize the relationship between P-gp expression and activity, NMGA-treated cells were subjected to calcein assay. The intracellular accumulation of calcein (which inversely correlates with P-gp activity) was significantly decreased after 48 h of treatment with 5, 50 and 500 nM NMGA (-25%, -28% and -23%, respectively), thereby indicating a higher efflux by P-gp and demonstrating the functional impact of NMGA-inducing effect. The reference P-gp inhibitor PSC833 increased intracellular calcein accumulation in both control and NMGA-treated cells, confirming the participation of P-gp in calcein-AM efflux (Fig 1d).

3.2. P-gp induction by NMGA is mediated by mPRα

We first performed a screening to identify the receptor triggering P-gp up-regulation. PR and PGRMC participation was evaluated using the pharmacological inhibitors RU 486 and AG-205, respectively. Since there is no mPR inhibitor available and considering that mPR is associated with G_i, we addressed its participation using the G_i protein inhibitor PTX. As represented in Fig 3a, RU 486 and AG-205 did not prevent NMGA-mediated P-gp up-regulation, thus ruling out the participation of PR and PGRMC, respectively. On the contrary, PTX prevented this effect, confirming the participation of a G_i protein-associated receptor and suggesting mediation by mPR.

The specific participation of mPR in NMGA was further evaluated. First, we confirmed by western blot studies that mPRα is the main mPR isoform expressed in both human liver and HepG2 cells, whereas the γ isoform was only detected using a high-sensitive chemiluminescence detection reagent (Fig 3b). Higher expression of mPRα in human liver and HepG2 cells was also verified through real time RT-PCR (Fig 3c). Next, we knocked down mPRα expression using small interfering RNA (siRNA). In this condition, mPRα protein expression was significantly decreased (-64%, Fig 3d) and the inducing effect of NMGA on P-gp was completely abolished, confirming the participation of this receptor (Fig 3e).

3.3. NMGA induces a biphasic modulation of cAMP levels and PKA activity

To further explore the molecular mechanism downstream the mPRα/G_i pathway, we evaluated the intracellular cAMP levels in NMGA-treated cells along a 24-h period. We found a significant decrease after 15 min of NMGA treatment (-51%, Fig 4a), suggesting that in our model mPRα is functionally linked to G_i and results in

inhibition of adenylate cyclase after ligand binding. Surprisingly, the initial decrease was followed by a significant increase in cAMP levels after 14 h of treatment (+131%, Fig 4a). This implies a novel falling-rising biphasic response in intracellular cAMP levels by NMGA.

Since increased cAMP levels usually lead to protein kinase A (PKA) activation, we evaluated PKA activity after NMGA treatment by assessing the level of phosphorylation of PKA substrates. A qualitative analysis of the representative western blots in Fig 4b showed an overall decrease in PKA-induced phosphorylation levels after 6 h of treatment followed by an overall increase after 22 h. These findings are consistent with an early decrease in PKA activity followed by a late PKA activation, agreeing well with the biphasic profile exhibited by cAMP levels (Fig 4a).

3.4. PKA is involved in NMGA-mediated P-gp up-regulation

Given that treatment with NMGA resulted in modulation of PKA activity and that this kinase has been already demonstrated to modulate *ABCB1* transcription [20, 36, 37], we further confirmed the participation of this kinase in the effect of NMGA on P-gp using a loss-of-function approach. Indeed, PKA inhibitors with different mechanism of action were used and, in all cases, P-gp induction was completely abolished (Fig 4c).

Participation of p38 and ERK1/2 in P-gp induction by NMGA was also possible since these MAP kinases were shown to be activated by mPRα [26, 38] and to regulate the expression of different genes [39]. Both inhibitors used to suppress p38 activity (SC68376) and to prevent ERK1/2 activation (PD98059) failed to prevent the increase in P-gp protein expression triggered by NMGA, ruling out the involvement of p38 and ERK1/2 kinases in this process (Fig 4c).

3.5. PDEs down-regulation is involved in late cAMP increase by NMGA

While initial cAMP decrease by NMGA treatment was expected due to G_i inhibitory action on adenylate cyclase, the subsequent increase in cAMP levels remains unexplained. Knowing that intracellular cAMP levels are determined not only by cAMP production by adenylate cyclase but also by cAMP degradation by PDEs, we repeated the cAMP measurement after 14 h of incubation with NMGA in the presence of PDE inhibitors. As seen in Fig 5a, the increase in intracellular cAMP level after 14 h of NMGA treatment was prevented by IBMX and Ro 20-1724, suggesting the participation of PDEs in this effect. In line with this result, we found a significant decrease in PDE activity towards cAMP after 11 h of NMGA treatment (-43%, Fig 5b). Finally, NMGA decreased mRNA expression of *PDE4D* (-36%, Fig 5c) by NMGA, the main subfamily of PDE involved in cAMP turnover in human liver and HepG2 cells, suggesting a participation of this enzyme in the altered cAMP turn-over described above.

Time-course analysis of P-gp mRNA expression exhibited a down-regulation at 6 h, agreeing well with decreased cAMP levels at earlier incubation times (Fig 5d). Conversely, no changes in the mRNA levels were observed at 48 h (Fig 5d), in line with normalization of cAMP levels after 24 h (Fig 4a). Taken together, these findings provide a rationale for the biphasic modulation of cAMP levels and for the associated induction of P-gp by NMGA.

4. Discussion

ABC transporters play a key role in the excretion and thus in the bioavailability of endo- and xenobiotics, including several therapeutic drugs [1]. In the current work, we assessed the effect of pharmacologically relevant concentrations of NMGA on the expression of the major hepatic drug efflux transporters. Our data indicates no modulation of MRP2, MRP3 or BCRP by NMGA. Since these transporters are involved in the efflux of several drugs and/or their conjugated metabolites, our study supports the safety of NMGA towards coadministration with substrates of these transporters. On the contrary, our results show a significant up-regulation of P-gp expression and activity by NMGA in two hepatic cell lines (Fig 1a and b). In this regard, an increase in the expression and activity of P-gp has been already related to several, in part life-threatening, drug-drug interactions [8, 40, 41]. For instance, healthy volunteers treated with rifampicin exhibited increased hepatic P-gp expression with higher biliary excretion of digoxin and concomitant reduction in its C_{max} and AUC [42]. In contrast to hepatic P-gp, in a pilot study using Caco-2 and LS180 intestinal cell lines, we did not observe changes in P-gp expression by NMGA (5-500 nM) (Tocchetti et al., unpublished results), thus suggesting a tissue-specific effect. Considering the long-term administration scheme of NMGA, sometimes through the whole reproductive age of the woman, its coadministration with other drugs is likely to occur. If a similar transporter up-regulation took place *in vivo*, higher hepatic clearance, reduced bioavailability and reduced therapeutic efficacy of coadministered drugs substrates of P-gp could be expected.

Our results showing an increase in *ABCB1* mRNA levels (Fig 1c) without changes in the mRNA half-life strongly suggest a transcriptional up-regulation by NMGA. A similar P-gp induction by progesterone was already demonstrated in porcine granulosa cells, being this effect mediated by the classical progesterone receptor

(PR) [19]. In our model, the PR antagonist RU 486 did not prevent P-gp induction by NMGA (Fig 3a), suggesting a PR-independent mechanism. These observations could be explained in terms of the differences in the agonists and cellular milieu between both studies. Nuclear receptor signaling is highly dependent on the function of coactivators and corepressors, which exhibit a cell-specific expression pattern and thus provide a rationale for tissue-selective effects following exposure to a particular ligand [43]. Therefore, PR ability to transactivate *ABCB1* promoter in granulosa cells may not necessary imply a similar transactivation in hepatic cells. Furthermore, activation of PR by NMGA in other tissues does not imply PR activation in hepatic cells. Interestingly, our results demonstrating a prevention of P-gp induction by PTX point to a G_i dependent mechanism (Fig 3a). Among progesterone receptors, mPRs are well-known for their association with Gi. Our results showing a prevention of P-gp induction by NMGA in mPRα knockdown cells (Fig 3e) further support the mediation by this membrane receptor. While mPR is well-known for mediating rapid and non-genomic effects of progestogens [18], genomic effects such as the regulation of cell proliferation and metastasis genes [44, 45] have also been described. To our knowledge this is the first report on a role of mPR in the regulation of drug transporters. Further studies should be performed to assess whether this action also takes place in other tissues of pharmaco-toxicological relevance (e.g. intestine, blood-brain barrier).

Membrane progesterone receptor (mPR) mechanism of action is based on the activation of G_i and further inhibition of adenylate cyclase, leading to decreased intracellular cAMP levels [17]. However, according to the literature, increased *ABCB1* transcriptional activity due to PKA activation is rather associated with increased intracellular levels of cAMP [36]. Intracellular levels of cAMP (and second messengers in general) are subject to a tight and dynamic regulation to better

respond to intracellular and extracellular challenges. Therefore, we performed a time-course study of intracellular cAMP levels after addition of NMGA. As expected from activation of the mPR/Gi pathway, a significant decrease was detected after 15 min (Fig 4a). Surprisingly, this initial decrease was followed by a significant increase peaking at 14 h (Fig 4a), clearly delineating a biphasic profile. Moreover, the analysis of PKA substrate phosphorylation (Fig 4b) as well as coincubations with 3 different inhibitors strongly indicate a mediation of NMGA effects by this kinase (Fig 4c).

Regulation of intracellular cAMP levels depends on the balance between synthesis and degradation. In this regard, phosphodiesterases (PDEs) play a key role [46]. Our results using PDE inhibitors (Fig 5a) indicate involvement of PDEs in the effect of NMGA on intracellular cAMP levels after 14 h of incubation. Indeed, in the absence of PDE inhibitors, NMGA leads to an increase in cAMP levels, being the outcome of reduced synthesis (due to adenylate cyclase inhibition) and subsequent inhibited degradation (probably due to PDE down-regulation). On the contrary, PDE inhibitors lead, as expected, to higher basal cAMP levels and a net decrease by NMGA, since only cAMP synthesis is subject to modulation. We further demonstrate decreased PDE activity (Fig 5b) and down-regulation of *PDE4D*, which encodes the main isoform mediating cAMP turnover in liver and HepG2 cells (Fig 5c). A feedback between cAMP levels and PDE mRNA expression was already reported, albeit in other models [47, 48]. Our results strongly suggest the decrease in *PDE4D* expression as a necessary step leading to cAMP increase and thus to P-gp up-regulation by NMGA.

In general, women are continuously exposed to varying levels of progestogens, both synthetic (e.g. NMGA and other contraceptive drugs) and physiological (progesterone). A similar alteration of P-gp expression by other physiological and synthetic mPR-activating progestogens, as described for NMGA, cannot be ruled out.

For instance, the contraceptive medroxyprogesterone acetate has been reported to signal through mPR in breast cancer cells [49]. Further studies should be performed to evaluate whether this interaction also takes place in hepatic cells and if, this way, P-gp expression can be modulated. Moreover, unpublished data from our group showed a similar mPRα-mediated P-gp up-regulation by progesterone in HepG2 cells (Tocchetti et al., unpublished results) at concentrations similar to those reported at the third trimester of pregnancy (100-1000 nM). Interestingly, a reduction in the bioavailability of drugs transported by P-gp like digoxin [50] and antiviral agents [51, 52] has been already demonstrated during this period of pregnancy and suggests also a pharmacological role of P-gp modulation by progestogens *in vivo*.

In conclusion, we here demonstrated an up-regulation of hepatic P-gp by the contraceptive progestogen NMGA. The effect takes place through a novel mechanism based on a mPR/G_i/adenylate cyclase-mediated down-regulation of intracellular cAMP levels coupled to subsequent compensatory *PDE4D* down-regulation and increase in cAMP levels, ultimately leading to PKA activation and transcriptional induction of *ABCB1*. Furthermore, we demonstrated a decrease in the intracellular accumulation of a P-gp model substrate. Clinical studies should be performed to assess whether a similar regulation takes place *in vivo*. If so, a decreased bioavailability and therapeutic efficacy of coadministered drugs can be expected.

Ethical standards

This work complies with the current rules of good scientific practice of the countries where it was performed.

5. Conflict of interest

The authors declare that they have no conflict of interest.

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7. References

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Figure legends

Fig 1. NMGA increases P-gp expression and activity. a. Effect of NMGA on P-gp protein expression in HepG2 cells. Cells were treated with 0.5-500 nM NMGA or vehicle for 48 h and then subjected to western blot analysis. **b.** Effect of NMGA on P-gp protein expression in Huh7 cells. Cells were treated with 0.5-500 nM NMGA or vehicle for 48 h and then subjected to western blot analysis. Equal amounts of total protein (12 µg) were loaded in each lane. Representative blots for P-gp and GAPDH (loading control) are shown. Data are presented as % of control group (C) and expressed as mean ± standard deviation (n = 3). *Different from C, $p < 0.05$. **c.** Effect of NMGA on *ABCB1* mRNA expression in HepG2 cells. Cells were treated with 5-500 nM NMGA or vehicle for 24 h. Then, *ABCB1* mRNA expression was assessed by real time RT-PCR and normalized to the expression of *HUPO*, used as a housekeeping gene. Data are presented as % of control group (C) and expressed as mean ± standard deviation (n = 3). *Different from C, $p < 0.05$. **d.** Effect of NMGA on P-gp activity in HepG2 cells. Relative fluorescence due to calcein accumulation was quantified by flow cytometry in control (C) and NMGA treated cells (5, 50 and 500 nM) and inversely correlated with P-gp activity. Experiments were performed in the presence or absence of the selective P-gp inhibitor PSC833 (10 µM). Data are presented as % of C without PSC833 and expressed as mean ± standard deviation (n = 3). a: $p < 0.05$ vs C, b: $p < 0.05$ vs all other groups.

Fig 2. NMGA does not modify MRP2, MRP3 and BCRP expression. Effect of NMGA on MRP2 **(a)**, MRP3 **(b)** and BCRP **(c)** protein expression in HepG2 cells. Cells were treated with 5-500 nM NMGA or vehicle for 48 h and then subjected to western blot analysis. Equal amounts of total protein (12 µg) were loaded in each lane. Representative blots for each target protein and GAPDH (loading control) are

shown. Data are presented as % of control group (C) and expressed as mean ± standard deviation (n = 3).

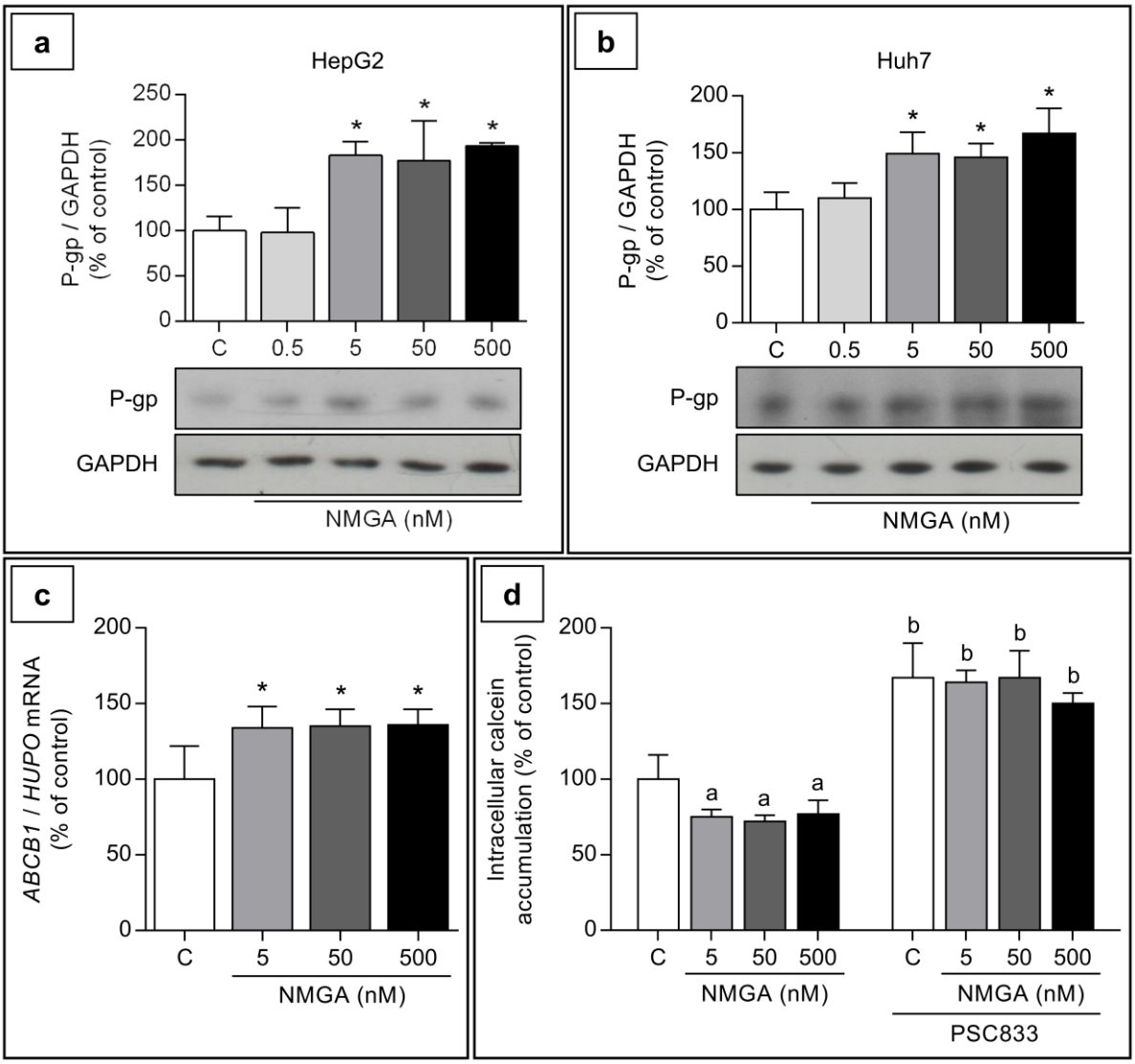
Fig 3. P-gp induction by NMGA is mediated by mPRα. a. G_i, but not PR or PGRMC, mediates NMGA-inducing effect. Cells were treated with 5-500 nM NMGA or vehicle in the presence of RU 486 (RU, 1 μM), AG-205 (AG, 20 μM) and *Pertussis* toxin (PTX, 200 ng/mL) as PR, PGRMC and G_i inhibitors, respectively. Equal amounts of total protein (12 μg) were loaded in each lane. Representative blots for P-gp and GAPDH (loading control) are shown. Data are presented as % of control group (C) and expressed as mean ± standard deviation (n = 3). *Different from the respective C, $p < 0.05$. **b.** HepG2 cells express mPRα. Expression of both α and γ isoforms of mPR was evaluated by western blot in human liver homogenates and HepG2 cell lysates. Equal amounts of total protein (15 μg) were loaded in each lane. While mPRα was easily detected in both samples, mPRγ was only detected in human liver by using a high-sensitive chemiluminescent detection kit. **c.** Comparison of expression of *PAQR7* (mPRα) and *PAQR5* (mPRγ) mRNA in human liver and HepG2 cells. The expression of the target genes was assessed by real time RT-PCR and normalized to the combined expression of *HUPO*, *β2-microglobuline* (*β2-mg*) and *RNA polymerase II* (*RPII*), used as housekeeping genes. For comparison purposes, data are presented as % of *PAQR7* expression in human liver and expressed as mean ± standard deviation. Data presented are result of two experiments. *Different from *PAQR7* expression in the same sample, $p < 0.05$. **d.** mPRα expression in transfected HepG2 cells. mPRα and GAPDH (loading control) were detected by western blot in lysates of HepG2 cells transfected either with 50 nM non-targeting control siRNA (C siRNA) or with 50 nM mPRα siRNA (pool of four

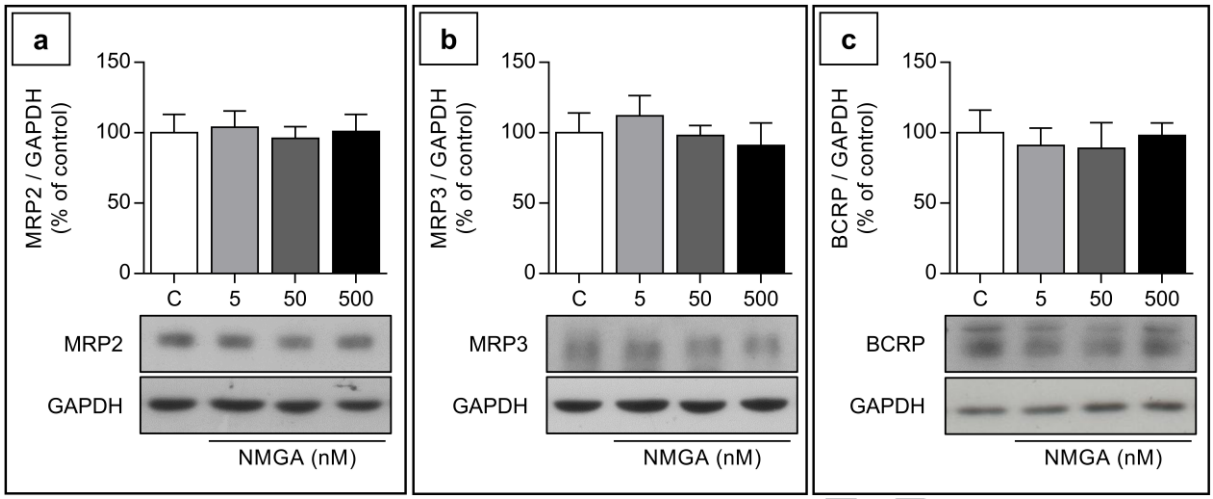
siRNA duplexes). Equal amounts of total protein (10 µg) were loaded in each lane. Data are presented as % of C siRNA and expressed as mean ± standard deviation (n = 3). *Different from C siRNA, $p < 0.05$. **e.** mPRα is involved in NMGA-mediated P-gp up-regulation. Equal amounts of total protein (12 µg) were loaded in each lane. Representative blots for detection of P-gp and GAPDH (loading control) proteins in lysates of HepG2 cells transfected either with 50 nM non-targeting control or with 50 nM mPRα siRNA are shown. Data on NMGA effects are presented as % of the respective C and expressed as mean ± standard deviation (n = 3). *Different from the respective C, $p < 0.05$.

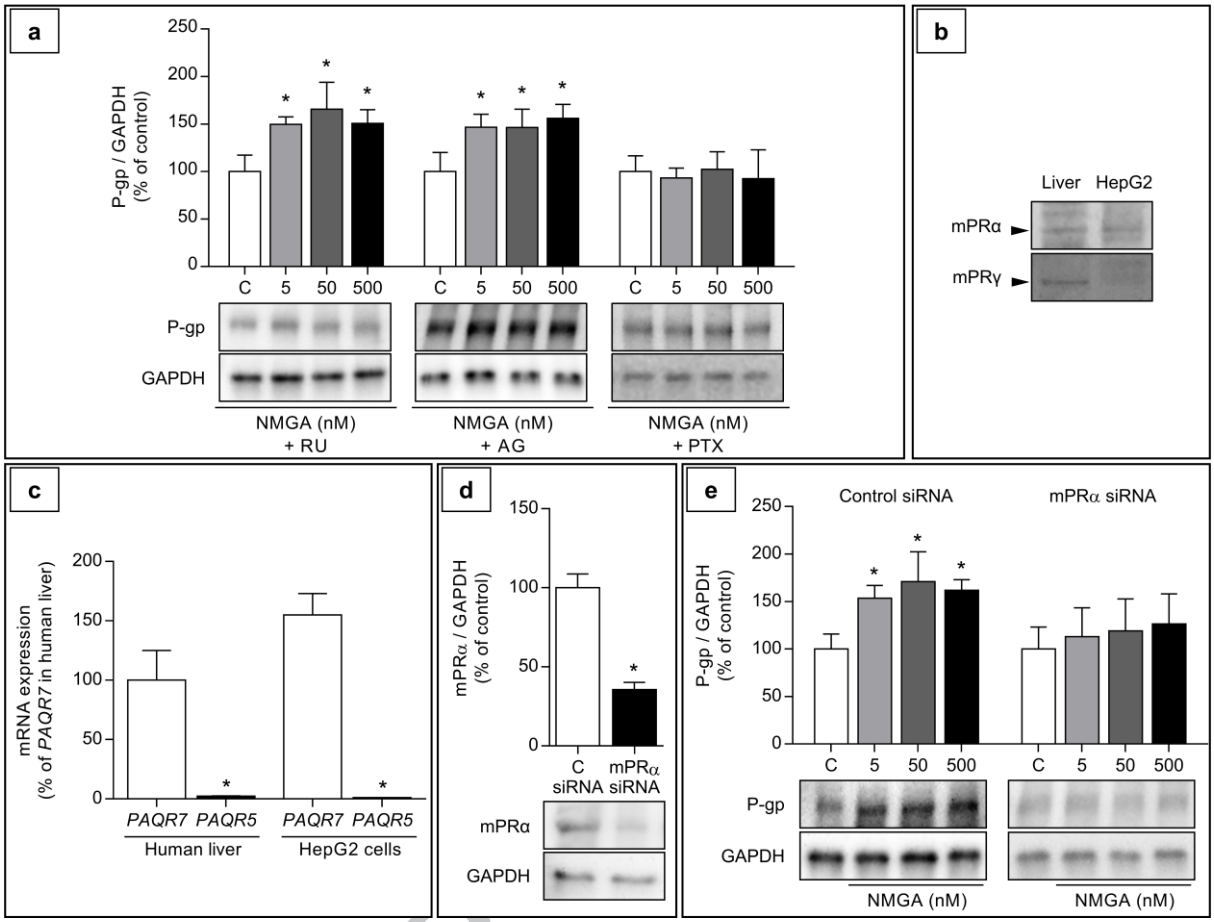
Fig 4. NMGA induces a biphasic modulation of cAMP levels and PKA activity, ultimately leading to increased P-gp expression. a. NMGA induces a biphasic modulation of intracellular cAMP levels. Time course of intracellular cAMP levels in NMGA-treated cells (dashed line) presented as % of control group (solid line). Cells were treated with 50 nM NMGA or vehicle during different time periods. Then, intracellular cAMP levels were measured using an ELISA kit. Data are expressed as mean ± standard deviation (n = 3). *Different from control, $p < 0.05$. **b.** NMGA induces a biphasic modulation of PKA activity. Phosphorylated PKA substrates were detected by western blot using anti-(P)-Ser/Thr PKA substrate. Arrows indicate proteins with altered phosphorylation status after NMGA treatment. Equal amounts of total protein (10 µg) were loaded in each lane. Uniformity of protein loading and transfer from gel to PVDF membrane was controlled with Ponceau S. **c.** PKA but not p38 or ERK1/2 is involved in NMGA-mediated P-gp up-regulation. Equal amounts of total protein (12 µg) were loaded in each lane. Representative blots for detection of P-gp and GAPDH (loading control) proteins in HepG2 cell lysates are

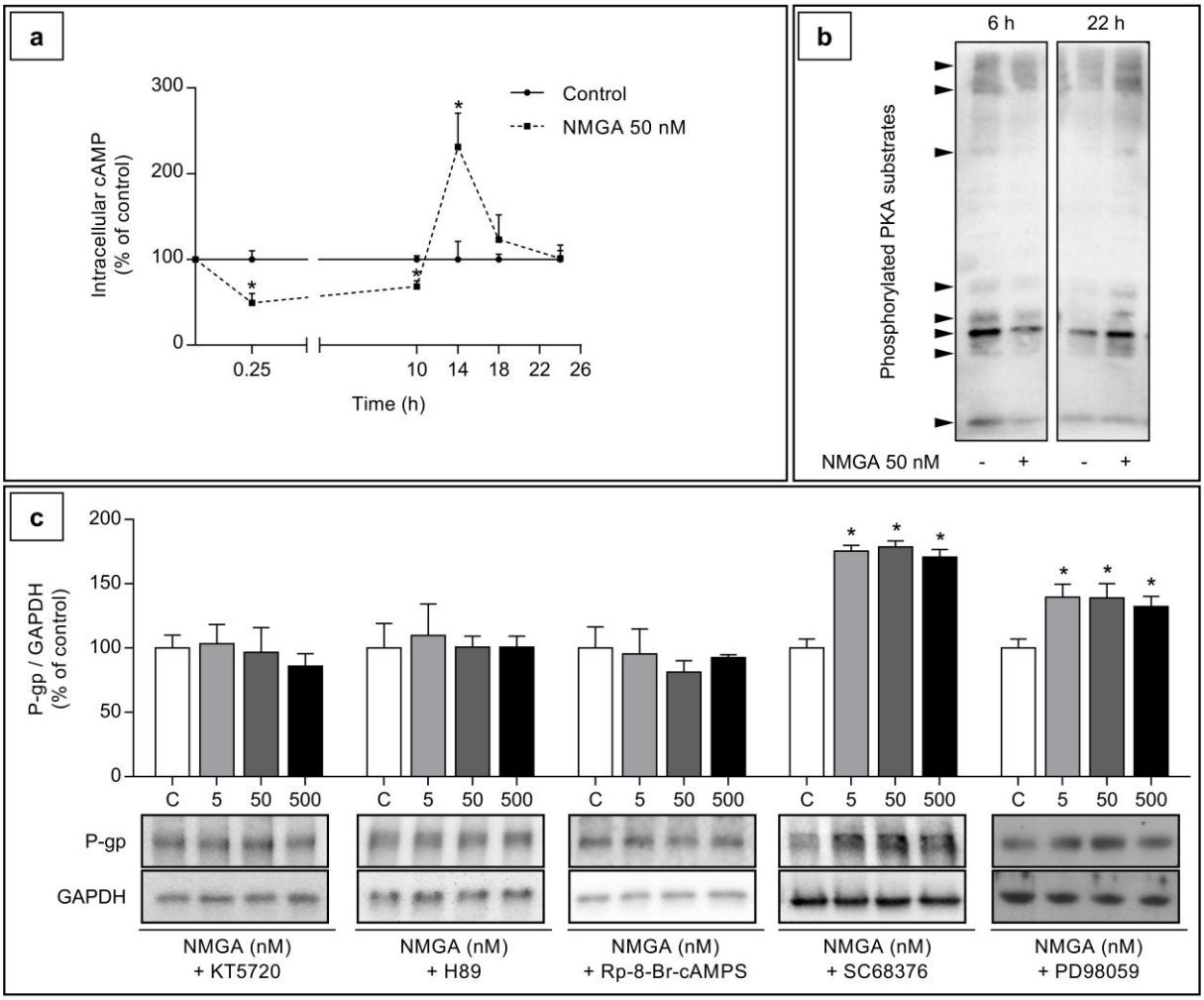
shown. 1 µM KT5720, 10 µM H89 and 100 µM Rp-8-Br-cAMPS were used as PKA inhibitors. 10 µM SC68376 and 20 µM PD98059 were used as inhibitors of p38 and ERK1/2 activation, respectively. Data are presented as % of control group (C) and expressed as mean ± standard deviation (n = 3).

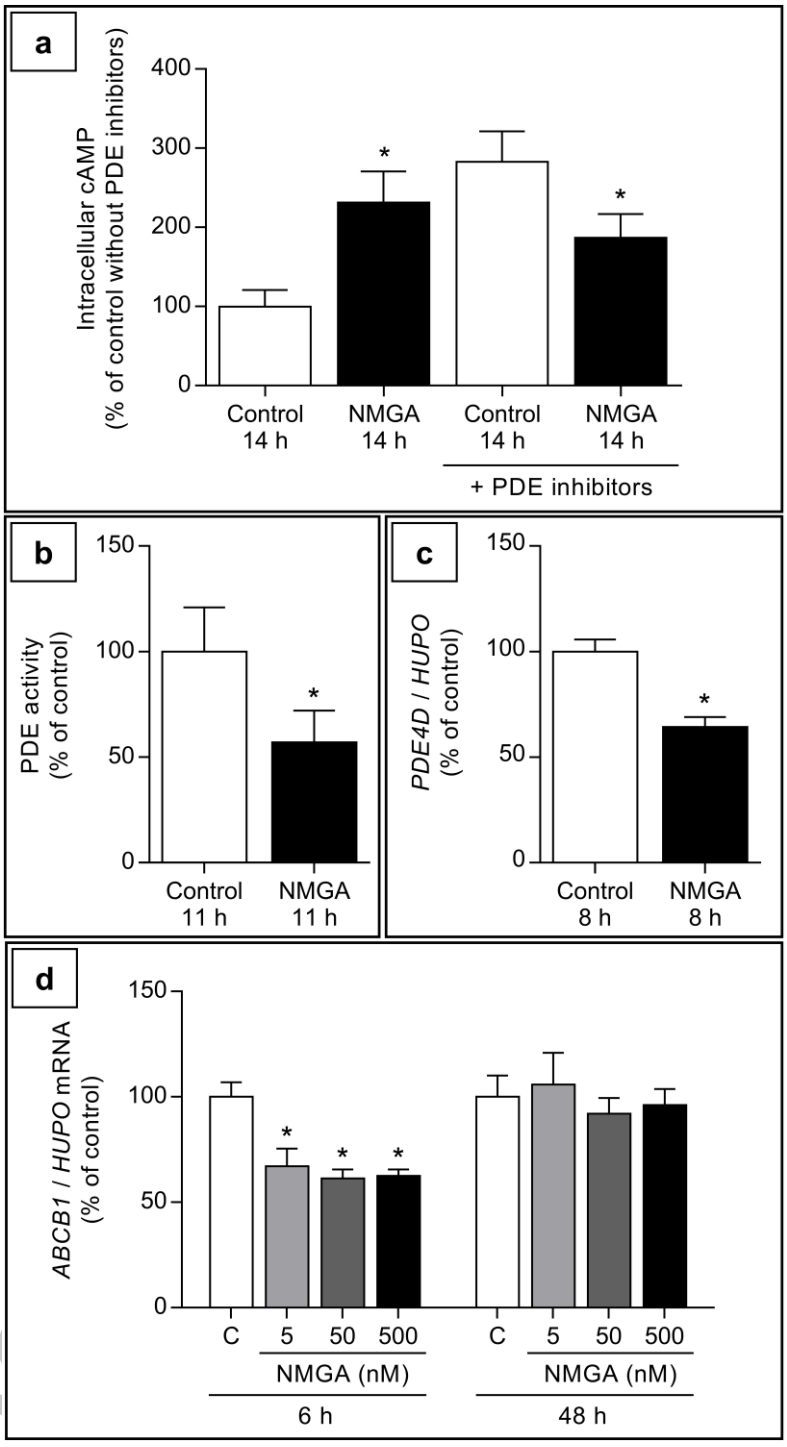
Fig 5. A decrease in PDE activity underlies NMGA-mediated cAMP increase. **a.** PDEs are involved in intracellular cAMP increase after 14 h of NMGA treatment. Cells were treated with 50 nM NMGA or vehicle during 14 h in the presence or absence of PDE inhibitors (500 µM IBMX and 100 µM Ro 20-1724). Then, intracellular cAMP levels were measured using an ELISA kit. Data are presented as % of control group without inhibitors and expressed as mean ± standard deviation (n = 3). *Different from the respective control, $p < 0.05$. **b.** NMGA decreases PDE activity after 11 h of treatment. Cells were treated with 50 nM NMGA or vehicle during 11 h. Then, PDE activity was determined in cell lysates using a colorimetric assay. Data are presented as % of control group and expressed as mean ± standard deviation (n = 3). *Different from control, $p < 0.05$. **c.** NMGA decreases *PDE4D* mRNA expression. Cells were treated with 50 nM NMGA or vehicle for 8 h. Then, *PDE4D* mRNA expression was assessed by real time RT-PCR and normalized to *HUPO* expression. Primers were designed to detect the mRNA of all PDE4D isoforms. **d.** Time-dependence of *ABCB1* mRNA regulation by NMGA. Cells were treated with 5-500 nM NMGA or vehicle for 6 or 48 h. Then, *ABCB1* mRNA expression was assessed by real time RT-PCR and normalized to the expression of *HUPO*, used as a housekeeping gene. All real time RT-PCR data are presented as % of control group (C) and expressed as mean ± standard deviation (n = 3-4). *Different from C, $p < 0.05$.











Graphical abstract