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Evidence for a Second Receptor for Prostacyclin on Human Airway Epithelial Cells that Mediates Inhibition of CXCL9 and CXCL10 Release

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Running Head: Prostacyclin receptor heterogeneity.

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ABBREVIATIONS: CRE, cAMP-response element; GRE, glucocorticoid response element; *E*/[A], concentration-effect; CXCL9, monokine induced by IFNγ; CXCL10, IFNγ-induced protein of 10 kDa; NTS, nucleus tractus solitarius; PGI_2, prostacyclin; PKA, cAMP-dependent protein kinase.

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ABSTRACT

Herein we provide evidence for the co-expression of two, distinct, prostacyclin (PGI_2) receptors (IP) on BEAS-2B human airway epithelial cells. IP-receptor heterogeneity initially was suggested by the finding that the rank orders of potency of PGI_2 and three structurally-similar analogs (taprostene, iloprost, 15-deoxy-TIC) for the inhibition of chemokine (CXCL9 and CXCL10) release and for transcriptional activation/augmentation of CRE and GRE luciferase reporters were distinct. Indeed, PGI_2, taprostene and iloprost activated both reporters whereas 15-deoxy-TIC was *inert*. Conversely, 15-deoxy-TIC, PGI_2 and taprostene (but *not* iloprost) suppressed chemokine release. Further experiments established that iloprost did not antagonize the inhibitory effect taprostene or 15-deoxy-TIC on chemokine output. Similarly, 15-deoxy-TIC failed to antagonize taprostene- and iloprost-induced reporter transactivation. Thus, iloprost- and 15-deoxy-TIC-induced responses were apparently mediated via pharmacologically-distinct receptors. In HEK-293 cells over-expressing the hrIP-receptor, 15-deoxy-TIC was considerably (>10,000-fold) less potent than iloprost and taprostene in promoting cAMP accumulation, yet in BEAS-2B cells these analogs were equipotent. IP-Receptor heterogeneity was also supported by the finding that the affinity of the IP-receptor antagonist, RO3244794, for the receptor mediating inhibition of chemokine release was approximately 10-fold lower than for the receptor mediating both transcriptional outputs. Finally, siRNAs directed against the IP-receptor gene, *PTGIR*, failed to block the suppression of chemokine output induced by taprostene and 15-deoxy-TIC, whereas taprostene- and iloprost-induced transcriptional responses were markedly attenuated. Collectively, these results indicate that PGI_2, taprostene and 15-deoxy-TIC suppress chemokine release from BEAS-2B cells by interacting with a novel IP-receptor, which we denote here as the "IP_2"-subtype.

Introduction

Prostacyclin (PGI_2) receptor (IP) heterogeneity is controversial. To date, cloning studies have not provided any substantive data indicative of IP-receptor subtypes. Similarly, the results of pharmacological investigations are inconclusive due to the paucity of selective ligands that can be used explicitly to classify responses mediated by IP-receptors (Wise and Jones, 2000). Interpreting data across species may also be problematic. Indeed, the atypically low sequence homology between IP-receptors (the human variant is only 73% identical at the amino acid level to the murine ortholog (Katsuyama et al., 1994)), could fundamentally alter the pharmacological behavior of *synthetic* IP-receptor agonists and antagonists and, potentially, create the false impression of receptor multiplicity.

Nevertheless, several studies conducted in the *same* species have provided evidence for multiple IP-receptors. Using the IP-receptor agonists, cicaprost and BMY 45778 (2-[3-[4-[4,5-di(phenyl)-1,3-oxazol-2-yl]-1,3-oxazol-5-yl]phenoxy]acetic acid), Wise et al., (1995) discriminated extra-neuronal from neuronal IP-receptor in the rat using neutrophils and enteric nerves in the colon as representative tissues respectively. In another study, Herbért and colleagues proposed the existence of three IP-receptors to account for the complex behavior of carbacyclin and iloprost at inhibiting vasopressin-induced water conductivity in rabbit cortical collecting ducts (Herbért et al., 1995). Unfortunately, the results of these studies are difficult to interpret unambiguously. For example, the variable sensitivity of the rat neutrophil and colon to BMY 45578 (Wise et al., 1995) could be a function of agonist efficacy (Wise and Jones, 2000). Thus, cicaprost could behave as a full agonist in both tissues, whereas BMY 45778 may only have sufficient efficacy in the colon to act as a partial agonist. In respect of the IP-receptor subtypes proposed by Herbért et al., (1995), there is evidence that the murine IP-receptor can couple to several G-proteins and, hence, multiple second messenger-generating enzymes (Namba et al., 1994). If the human ortholog is similarly promiscuous, the need to invoke multiple IP-receptors is negated.

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IP-Receptor subtypes in the rodent CNS have also been proposed. Using the radiolabelled IP-receptor agonists, [^{3}H]isocarbacyclin and [^{3}H]iloprost, Takechi et al., (1996) identified two, apparently distinct, binding sites in rat brain. Specifically, [^{3}H]isocarbacyclin bound to a site in the thalamus and nucleus tractus solitarius (NTS) with similar K_ds. In contrast, [^{3}H]iloprost discriminated between these brain regions having 23-fold higher affinity for sites in the NTS relative to the thalamus. Based on these data, it was concluded that the high affinity and relatively low affinity [^{3}H]iloprost binding sites identified in the NTS and thalamus respectively represented different IP-receptor subtypes (Takechi et al., 1996). Additional evidence for this taxonomy is that the 15***R*** epimer of 16-*m*-tolyl-17,18,19,20-tetranor isocarbacyclin (15***R***-TIC) (Suzuki et al., 1996) and the achiral 15-deoxy derivative (15-deoxy-TIC; Fig. 1) were significantly more potent at binding to the site in the thalamus when compared with the NTS (Suzuki et al., 1999, Takechi et al., 1996, Watanabe et al., 1999). In considering the implications of these data, at least four issues arise that are potential causes for concern. First, the assignation of the two binding sites in the CNS as distinct IP subtypes is critically dependent upon the exclusive interaction of [^{3}H]isocarbacyclin with the IP-receptor. However, [^{3}H]isocarbacyclin has affinity for the EP_2- and EP_3-receptor subtypes (Dong et al., 1986, Kiriyama et al., 1997) and so this assumption may be flawed. Second, iloprost has appreciable affinity at EP_1- and EP_4-subtypes (Jones et al., 2009, Wilson et al., 2004, Wilson and Giles, 2005). Thus, the radioligand binding data may not reflect an exclusive interaction of iloprost with IP-receptors. Third, the selectivity of 15***R***-TIC and 15-deoxy-TIC for the known prostanoid receptors is undefined. Fourth, the activity of naturally-occurring prostanoids at their cognate receptors requires a hydroxyl-bearing chiral centre at position C15 that, typically, is in the ***S***-configuration (Fig. 1; Monneret et al., 2003). The potent activity of 15***R***-TIC and 15-deoxy-TIC is, therefore, difficult to explain unless the 15***S***-configuration is not critical for IP-receptor agonism (as has been shown for DP_2-receptor activation (Monneret et al., 2003)), or that [^{3}H]isocarbacyclin and

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[^{3}H]iloprost label a site in the CNS that is distinct from known prostanoid receptors. Thus, IP-receptor heterogeneity is neither established nor disproved.

In the present study, using a panel of IP-receptor agonists (PGI_2, iloprost, taprostene, 15-deoxy-TIC; Fig. 1) and the surmountable, competitive and selective IP-receptor antagonist, RO3244794 (Bley et al., 2006), we now provide clear functional evidence for the co-expression of two IP-receptor subtypes on the human airway epithelial cell line, BEAS-2B, that mediate distinct functional responses.

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Materials and Methods

Reagents. 15-Deoxy-TIC (15-deoxy-16-(*m*-tolyl)-17,18,19,20-tetranorisocarbacyclin) was synthesized using a novel ω-side chain addition strategy as detailed previously (Sheddan et al., 2007, Sheddan and Mulzer, 2005). BWA A868C ([3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-ydroxyethylamino) hydantoin]), PGE_2 and iloprost were purchased from Cayman Chemicals (Ann Arbor, MI). ONO-AE1-259 ((*Z*)-7-[(1*R*,2*S*,3*R*,5*R*)-5-chloro-3-hydroxy-2-[(*E*,4*S*)-4-hydroxy-4-(1-prop-2-enylcyclobutyl)but-1-enyl]cyclopentyl]hept-5-anoic acid) and RO3244794 (*R*-3-(4-fluorophenyl)-2-[5-(4-fluorophenyl)-benzofuran-2-yl-methoxycarbonyl-amino] propionic acid) were provided by ONO Pharmaceuticals (Osaka, Japan) and Roche (Palo Alto, CA) respectively. L-798,106 (5-bromo-2-methoxy-*N*-[3-(2-naphthalen-2-yl-methylphenyl)acryloyl]-benzene sulphonamide) and L-161,982 (5-butyl-2,4-dihydro-[[2'-[*N*-(5-methyl-2-thiophene-carbonyl)sulphamoyl]biphenyl-4-yl]methyl]-2-[(2-trifluoromethyl) phenyl]-1,2,4-triazol-3-one) were donated by Merck Frosst (Montreal, Canada). All other reagents were from Sigma/Aldrich (Oakville, Ontario, Canada).

CRE And GRE Luciferase Reporters. Stable transfection was used to generate CRE and GRE BEAS-2B reporter cell lines (Chivers et al., 2004, Meja et al., 2004). Luciferase activity was measured by luminometry and expressed as a fold induction relative to unstimulated cells.

Cell Culture. BEAS-2B cells and HEK-293 cells over-expressing the hrIP-receptor (IP*R*-HEK), DP_1-receptor (DP_1*R*-HEK), EP_2-receptor (EP_2*R*-HEK) and EP_4-receptor (EP_4*R*-HEK) subtypes were cultured as described previously (Ayer et al., 2008. Wilson et al., 2009).

Measurement of Chemokines. CXCL9 (monokine induced by γ-interferon) and CXCL10 (IFNγ-induced protein of 10 kDa) were measured by sandwich ELISA (Human DuoSet®; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

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Transfection of Cells with siRNAs. RNAiMax® (Invitrogen), the siRNA of interest (10 nM; Table 1) and relevant controls were added to subconfluent (~70%), growth-arrested BEAS-2B cells as described previously (Wilson et al., 2009). Effective "knockdown" of the IP-receptor protein was assessed by western blotting using a murine monoclonal IP-receptor antibody (cat# AT3484a) from Abgent (San Diego, CA). IP*R*-HEK cells were used as a positive control.

Measurement of cAMP. Cells were pre-treated (30 min) with the PDE3 and PDE4 inhibitors, siguazodan (10 μM) and rolipram (10 μM) respectively. IP-receptor agonists and/or antagonists were then added for an additional 45 min, a time when steady state levels were achieved. cAMP was quantified by EIA (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions.

Infection of Cells with Adenovirus Vectors. Sub-confluent BEAS-2B cells were infected (>90% efficiency) with an adenovirus vector (Ad5.CMV.PKIα) and a null control (Ad5.CMV.Null) encoding the α-isoform of cAMP-dependent protein kinase (PKA) inhibitor as described before (Meja et al., 2004).

Curve Fitting and the Estimation of Antagonist Affinity. Monophasic agonist concentration-effect (*E*/[A]) curves were fitted by least-squares, non-linear iterative regression to the following form of the Hill equation (Prism 4®, GraphPad Software Inc, San Diego, CA):

$$E = E_{min} + \frac{(E_{max} - E_{min})}{1 + 10^{(p[A]_{50} - p[A])^{n}}} \qquad (1)$$

where E is the effect, E_{min} and E_{max} are the lower and upper asymptote (i.e. the basal response and maximum agonist-induced response respectively), p[A] is the log molar concentration of agonist,

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$p[A]_{50}$ is a location parameter equal to the log molar concentration of agonist producing $(E_{max}\text{-}E_{min})/2$ and n is the gradient of the E/[A] curve at the $p[A]_{50}$ level.

The affinity of RO3422794 was estimated by least-squares, non-linear regression using a modification (Waud et al., 1978) of the Hill and Gaddum/Schild equations (Lazareno and Birdsall, 1993). Each IP-receptor agonist was employed at a fixed sub-maximal concentration (~ $p[A]_{95}$) in the absence and presence of increasing concentrations of RO3244794. For this modified Schild analysis, knowledge of the precise location of the agonist E/[A] curve is also required and this was determined in parallel on the same batch of cells. Each pair of E/[A] curves (i.e. the control E/[A] curve and the E/[A] curve constructed in the presence of increasing concentrations of RO3244794) was fitted simultaneously to Equation (2). Thus,

$$E = E_{min} + \left(\frac{(E_{max} - E_{min})}{1 + \left(\frac{10^{p[A]_{50}} \left[1 + \left(\frac{[B]}{10^{-pA_2}} \right)^S \right]}{[A]} \right)^n} \right) \qquad (2)$$

where [A] and [B] are the molar concentration of agonist and RO3244794 respectively, S is the Schild slope factor, which indicates the nature of antagonism, and pA_2 is the affinity of the antagonist when S = 1, which is equivalent to the pK_b. To determine whether S deviated significantly from unity, the pair of E/[A] curves that made up an individual experiment was fitted globally to Equation (2) under two conditions: one where S was constrained to a constant equal to 1 and the other where it was a shared value for all data sets. The F-test was applied to determine which equation gave the best fit, which was used in the final analysis.

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Statistics. Data points, and values in the text and figure legends, represent the mean ± s.e. mean of *N* independent determinations. Where appropriate, data were analyzed statistically using Student's paired *t*-test or by one-way ANOVA/Bonnferoni's multiple comparison test. The null hypothesis was rejected when $p < 0.05$.

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Results

Selectivity of 15-deoxy-TIC, iloprost and taprostene for the DP_1-, EP_2- and EP_4-receptor subtypes. To establish the selectivity of the IP-receptor agonists shown in figure 1 for Gs-coupled prostanoid receptors, cAMP accumulation was measured in HEK-293 cells over-expressing the DP_1-, EP_2- and EP_4-subtypes. As shown in figure S1*A*-*C*, 15-deoxy-TIC was inactive at each of these receptor at concentrations 100-1000-times higher than maximally effective concentrations of both the natural ligands and representative synthetic agonists. Iloprost, similarly, did not elevate cAMP in hrEP_2-HEK cells but did display some activity at the DP_1- and EP_4-subtypes (Fig S1*D*- *E*). Indeed, iloprost was a potent ($p[A]_{50}$ ~ 11.4), albeit partial, DP_1-receptor agonist (Fig. S1*D*). We have reported, previously, that taprostene does not increase cAMP in hrEP_2*R*-HEK-293 cells but is a weak agonist of very low potency in HEK-293 cells over-expressing the DP_1 and EP_4-receptor subtypes (see figure 2 in Wilson et al., 2009). Collectively, these data show that iloprost and 15-deoxy-TIC are selective IP-receptor agonists but may, nevertheless, have the ability to activate DP_1- and EP_4-receptors at high concentrations. Accordingly, unless stated otherwise, all experiments were performed under conditions of concurrent DP_1 (BWA 868C 1 μM)- and EP_4 (L-161,982, 500 nM)-receptor blockade. The EP_3-receptor antagonist, L-798,106 (100 nM), was also included in all experiments to eliminate the possible contribution of splice variants of the EP_3-receptor that can couple to adenylyl cyclase (Kotani et al., 1995).

Effect of IP-receptor Agonists on Chemokine Release and on Reporter Activity. The effects of the IP-receptor agonists shown in figure 1 were examined on two classes of functional response in BEAS-2B cells: (i) inhibition of IFNγ (100ng/ml; $p[A]_{85}$)-induced CXCL9 and CXCL10 release, and (ii) activation of a CRE-reporter and augmentation of a dexamethasone (1 μM)-driven GRE-reporter (Ayer et al., 2008, Chivers et al., 2004, Kaur et al., 2008, Meja et al., 2004).

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Taprostene, PGI_2, iloprost and 15-deoxy-TIC inhibited the release of CXCL9 and CXCL10 in a concentration-dependent manner (Fig. 2*A*-*H*; Table 2). In the presence of BWA 868C, L-798,106 and L-161,982 neither the $p[A]_{50}$ nor the E_{max} values of taprostene, PGI_2 and 15-deoxy-TIC were significantly affected (Table 2). In contrast, the inhibitory effects of iloprost were abolished (Fig. 2*C* & *G*) indicating that these responses were *not* mediated by the IP-receptor subtype. Indeed, further studies with BWA 868C and L-161,982 established that these effects of iloprost were mediated by both DP_1- and EP_4-receptors (see Fig. S2 for CXCL10 data and Wilson et al., 2004, Wilson et al., 2005). Taprostene, PGI_2 and iloprost also promoted CRE-dependent transcription and augmented dexamethasone-stimulated GRE-reporter activity. Consistent with the chemokine release data described above, the concentration-effect (*E*/[A]) relationships that described these responses were not modified by BWA 868C, L-798,106 and L-161,982 (Fig. 2*I-K*, *M-O*; Table 2). In contrast to its inhibitory effect on chemokine release, 15-deoxy-TIC was inactive on both reporter constructs (Fig. 2*L* & *P*). Thus, these ligands were categorized into those that (i) inhibited chemokine output *and* promoted CRE- and GRE-dependent transcription (PGI_2, taprostene); (ii) activated the reporters only (e.g. iloprost); and (iii) suppressed chemokine output only (e.g. 15-deoxy-TIC). Accordingly, the rank orders of agonist potency for the inhibition of chemokine release (15-deoxy-TIC > taprostene > PGI_2) and for the activation of both reporters (iloprost > taprostene > PGI_2) were distinct.

Iloprost and 15-deoxy-TIC do not Recognize the same Receptor on BEAS-2B Cells. Pre-treatment of BEAS-2B cells with a concentration (1 μM) of iloprost that maximally activated both the CRE and GRE reporters did not antagonize the inhibitory effects 15-deoxy-TIC or taprostene on CXCL9 or CXCL10 release (Figs. 3*A* & *B*, *S3A* & *B*). Similarly, on CRE- and GRE-dependent transcription, a concentration of 15-deoxy-TIC (1 μM) that maximally suppressed chemokine release failed to antagonize CRE or GRE reporter activation induced by taprostene and iloprost (Fig. 3*C* & *D*, Fig S3*C* & *D*).

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RO3244794 Antagonizes Iloprost- and 15-Deoxy-TIC-induced Responses with Different Affinities. Typically, the affinity of an antagonist for a given receptor is invariant within species. Accordingly, a modification (Waud et al., 1978) of the Hill and Gaddum/Schild equations (Lazareno and Birdsall, 1993) was used to determine if there were differences in the affinity of the competitive, insurmountable IP-receptor antagonist, RO3244794 (Bley et al., 2006; Jones et al., 2008) for the receptor(s) mediating iloprost-, taprostene and 15-deoxy-TIC-induced responses. RO3244794 (10 pM to 100 nM) had no effect on IFNγ-induced chemokine release from BEAS-2B cells at any concentration examined (data not shown). In contrast, RO3244794 added to cells 30 min prior to a fixed, sub-maximal concentration of taprostene (1 μM) antagonized the inhibitory effect of taprostene on IFNγ-induced CXCL9 and CXCL10 release in a concentration-dependent manner (Fig. 4*A* & *C*). Enumeration of the Schild slope factor, S, by simultaneously fitting to Equation (2) each pair of RO3244794 and taprostene *E*/[A] curves, which were constructed simultaneously (see **Methods**), indicated that this parameter did not deviate significantly from unity for either chemokine. Thus, RO3244794 behaved as a surmountable competitive antagonist (Neubig et al., 2003) from which pK_bs of 10.3 (CXCL9) and 10.2 (CXCL10) were derived (Fig. 4*A* & *C*). Similar affinities were obtained when 15-deoxy-TIC was used as agonist (pK_bs: CXCL9 = 9.90; CXCL10 = 9.79; see Fig. 4*B* & *D*).

On CRE- and GRE-dependent transcription, RO3244794 also behaved competitively (Fig. 4*E*-*H*). However, while the affinity of RO3244794 was the same irrespective of whether taprostene (pK_bs: CRE = 8.87; GRE = 9.10) or iloprost (pK_bs: CRE = 9.06; GRE = 9.53) were used as agonist, it was, nevertheless, ~10-fold lower than the pK_b determined from the chemokine release experiments. Thus, these data suggest that 15-deoxy-TIC and iloprost bind to different receptors.

15-Deoxy-TIC is a Low Potency Agonist Relative to Iloprost in Promoting cAMP Accumulation in IP*R*-HEK-293 Cells, but is Equipotent with Iloprost in BEAS-2B Cells. In HEK-293 cells over-

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expressing the human IP-receptor, taprostene, iloprost and 15-deoxy-TIC promoted cAMP accumulation in a concentration-dependent manner (Fig. 5*A*). 15-deoxy-TIC was a full agonist (α = 1.05 relative to iloprost) in this system with a $p[A]_{50}$ of 8.55 ± 0.01. However, relative to iloprost ($p[A]_{50}$ = 13.27 ± 0.02) and taprostene ($p[A]_{50}$ = 12.67 ± 0.2), 15-deoxy-TIC was greater than 52,000- and 13,000-fold less potent, respectively. In contrast, iloprost, taprostene and 15-deoxy-TIC increased the cAMP concentration in BEAS-2B cells in a concentration-dependent manner with similar $p[A]_{50}$ values (8.63 ± 0.05, 8.00 ± 0.01 and 8.70 ± 0.02 respectively; Fig. 5*B*). Thus, in this system 15-deoxy-TIC was equipotent with iloprost and 5-times more potent than taprostene.

Effect of Iloprost and 15-Deoxy-TIC on cAMP Levels. At their maximally effective concentrations, iloprost and 15-deoxy-TIC were almost equi-effective at increasing cAMP in BEAS-2B cells (Fig. 5*B* & *C*). Despite inducing comparable response, only iloprost activated the CRE and GRE reporter cells (Fig. 2*K* & *O*), whereas only 15-deoxy-TIC suppressed chemokine release (Fig. 2*D* & *H*). In contrast, taprostene, increased cAMP maximally by an amount that was approximately 1.8- and 2.5-fold greater than that produced by iloprost and 15-deoxy-TIC respectively (Fig. 5*B & C*), and was active in both functional assays (Fig. 2*A, E, I* & *M*). When examined in combination, iloprost (100 nM) and 15-deoxy-TIC (100 nM) increased the cAMP concentration in an additive manner and by an amount that was equivalent in magnitude to the maximum taprostene (1 μM)-induced response (Fig. 5*C*). However, neither iloprost nor 15-deoxy-TIC increased further the cAMP content in BEAS-2B cells produced by a maximally effective concentration of taprostene (Fig. 5*C*). Thus, in BEAS-2B cells iloprost and 15-deoxy-TIC apparently generated distinct pools of cAMP that regulated different functional responses. In the presence of RO3244794 (1 μM), taprostene-, iloprost- and 15-deoxy-TIC-induced cAMP accumulations were abolished (Fig. 5*D*).

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Iloprost and 15-deoxy-TIC act via cAMP-dependent Protein Kinase Cascades. It is well established that the biological actions of cAMP are mediated by several effectors. Thus, additional studies were performed to identify the down-stream target(s) of the cAMP generated by iloprost and 15-deoxy-TIC. Infection of BEAS-2B cells with the adenovirus vector, Ad5.CMV.PKIα (MOI = 40), which directs over-expression of a selective inhibitor of PKA (Meja et al., 2004), prevented the ability of 15-deoxy-TIC to suppress IFNγ-induced CXCL9 and CXCL10 release under conditions where an empty vector, Ad5.CMV.Null, was inactive (Fig. 6*A* & *B*). Similarly, the transactivation of the CRE and GRE reporters by iloprost was blocked in Ad5.CMV.PKIα- but not Ad5.CMV.Null-infected cells (Fig. 6*C* & *D*). Thus, in BEAS-2B cells, these responses induced by both iloprost and 15-deoxy-TIC required the activation of cAMP/PKA signalling cascades.

"Silencing" the IP-Receptor Gene with siRNAs Selectively Inhibits Iloprost- but not 15-Deoxy-TIC-induced Responses. Lipid-mediated transfection of BEAS-2B cells with siRNAs (PTGIR-1, PTGIR-2; see Table 1 for sequences) directed against *PTGIR* and all reported putative splice variants thereof (www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?exdb=AceView&db=36a&term=ptgir) significantly reduced the expression of the variously glycosylated IP-receptor (Smyth et al., 1996) assessed by western blotting (Fig 7A, lane 3), whereas the transfection lipid, RNAiMax, and a universal negative control (UC) oligonucleotide (AllStars, Qiagen) were inactive (Fig. 7*A*). Functionally, both oligonucleotides inhibited (by >76%) taprostene (1 μM)- and iloprost (100 nM)-induced CRE and GRE reporter activation under conditions where the same responses evoked by PGE_2 (1 μM) were preserved (Fig. 7*B* & *C*; see Fig. S4*A* & *B* for control siRNA data). The PTGIR-1 siRNA duplex was the most effective (Fig. S4*A* & *B*), with the enhancement of GRE-reporter activity being particularly sensitive (~99% inhibition) to this intervention (Fig. 7*C*). In contrast, the *E*/[A] curves that described the inhibitory actions of taprostene and 15-deoxy-TIC on chemokine release were unaffected in cells transfected with PTGIR-1 (Fig. 7*D* - *G*) and PTGIR-2 (Fig. S4*C* & *D*).

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Taprostene- and iloprost-induced cAMP accumulations also were inhibited (45.4 ± 0.5% and 63.7 ± 1.4% respectively) in cells transfected with PTGIR-1 siRNAs (Fig. 8*A* & *B*), whereas the increments in cAMP produced by 15-deoxy-TIC and PGE_2 (a negative control) were unaffected (Fig. 8*C* & *D*). The transfection lipid, RNAiMax, and siRNAs that target the genes encoding lamin A/C, GFP as well the UC oligonucleotide had no effect on any of the functional outputs studied (Figs. 7*B-E*; 8 & S4*A-D*).

To exclude the possibility that the generation of the stable transfectants in BEAS-2B cells perturbed the ability of the IP-receptor to recognize agonists, the effects of the PGI_2 analogs on chemokine release and on the CRE reporter were determined concurrently in the same cell cultures. As shown in figure S5*A-C*, CRE reporter cells responded to taprostene, iloprost and 15-deoxy-TIC in the absence and presence of PTGIR-1 in an identical manner to non-transfected cells (*cf*. Fig. 2).

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Discussion

In the present study, we provide evidence for the co-expression of two IP-receptors in the human airway epithelial cell line, BEAS-2B. It is important to restate that these studies were performed in the presence of L-798,106, L-161,982 and BWA 868C to eliminate any contribution of EP_3-, EP_4- and DP_1-receptors, which can couple, positively to adenylyl cyclase. Preliminary studies also firmly established that none of the IP-receptor agonists studied in the present study activated the hrEP_2-receptor expressed in HEK-293 cells.

IP-receptor multiplicity initially was suggested by differences in the rank orders of potency of PGI_2 and three structurally-related agonists for the inhibition of chemokine release (15-deoxy-TIC > taprostene > PGI_2 (iloprost was inactive at 1 μM)) and the activation of CRE- and GRE-dependent transcription (iloprost > taprostene > PGI_2 (15-deoxy-TIC was inactive at 1 μM)). However, the classification of receptors using agonists alone is problematic because variability in potency orders between functional responses, even in the same cell type, may reflect differences in efficacy rather than receptor subtypes. Indeed, an alternative interpretation is that 15-deoxy-TIC has high affinity for the IP-receptor, but low efficacy in driving CRE- and GRE-dependent transcription when compared to the inhibition of chemokine output. If this explanation is correct, then 15-deoxy-TIC should behave as an IP-receptor antagonist. As shown in figure 3, a high concentration of 15-deoxy-TIC that maximally suppressed chemokine release failed to produce a dextral displacement of the taprostene and iloprost *E*/[A] curves that described the activation of the two reporter constructs. Thus, the affinity of 15-deoxy-TIC for the IP-receptor that is sensitive to both taprostene *and* iloprost is, at best, weak (> 1 μM). This conclusion was corroborated by the finding that 15-deoxy-TIC was ~52,000- and ~13,000-times less potent than iloprost and taprostene respectively in promoting cAMP accumulation in HEK-293 cells over-expressing the hrIP-receptor. Differences in efficacy could also account for the inability of iloprost to suppress chemokine release. However, iloprost did not display measurable affinity for the 15-deoxy-

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TIC-sensitive IP-receptor subtype as evinced from its inability to antagonize the inhibition of chemokine generation (Fig. 3). Collectively, therefore, these pharmacological data suggest the presence of two taprostene (and PGI_2)-sensitive receptors on BEAS-2B cells, which are denoted here as IP_1-(iloprost-sensitive) and "IP_2" (15-deoxy-TIC-sensitive) subtypes.

Further studies using a surmountable, competitive IP-receptor antagonist, RO3244794 (Bley et al., 2006), that has a high affinity (pK_b = 9.24 - 8.50) and selectivity for the IP-receptor subtype (Bley et al., 2006, Jones et al., 2009) provided additional evidence for IP-receptor heterogeneity. Thus, RO3244794 antagonized taprostene- and iloprost-induced CRE- and GRE-reporter activation with an affinity ($pK_b \sim 9$) consistent with an interaction with the known, cloned IP-receptor (IP_1) (Katsuyama et al., 1994). RO3244794 also antagonized the ability of taprostene and 15-deoxy-TIC to suppress CXCL9 and CXCL10 release. However, in these experiments, RO3244794 had significantly higher affinity ($pK_b \sim 10$) suggesting that chemokine output was regulated by a receptor ("IP_2") that was distinct from the IP_1-subtype recognized by iloprost and taprostene in the reporter cells. There are limited data with 15-deoxy-TIC from which pharmacodynamic parameters can accurately be derived. Nevertheless, applying the "IP_1/IP_2" nomenclature introduced above, our results are supported by the finding that 15-deoxy-TIC is a weak inhibitor of platelet aggregation (IP_1-mediated) relative to isocarbacyclin (Suzuki et al., 1996, Suzuki et al., 1999). Conversely, 15-deoxy-TIC rescues gerbil cultured hippocampal neurons from oxygen-induced apoptosis ("IP_2"-mediated) under conditions where iloprost is inactive (Satoh et al., 1999). This classification is also supported by Takechi et al., (1996) who suggested the existence of a second IP-receptor subtype in the rat CNS based on radioligand binding data (see **Introduction**).

Iloprost, taprostene *and* 15-deoxy-TIC elevated cAMP in BEAS-2B by a mechanism that was abolished by the IP-receptor antagonist, RO3244794. Moreover, adenovirus-delivery of PKIα, a highly

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selective inhibitor of PKA (Meja et al., 2004), to these cells similarly blocked the effects of 15-deoxy-TIC and iloprost on chemokine release and reporter activity respectively. Thus, the "IP_2"-receptor as well as the well characterized IP_1-subtype mediated these functional effects by activating classical cAMP/PKA cascades.

At maximally effective concentrations, cAMP accumulation induced by taprostene was 2.5- and 1.8-fold higher than that elicited by 15-deoxy-TIC and iloprost respectively. These data led us to speculate that the increments in cAMP induced by iloprost and 15-deoxy-TIC were due to selective agonism of IP_1- and "IP_2"-receptors respectively, whereas taprostene activated both subtypes resulting in an additive cAMP response. Indeed, when used in combination at their maximally-effective concentrations, iloprost and 15-deoxy-TIC produced cAMP signals that were the sum of the increments produced by both drugs alone and equivalent in magnitude to the taprostene-induced response.

To consolidate the evidence that BEAS-2B cells express two, pharmacologically-distinct IP-receptors, the gene encoding the IP_1-subtype, *PTGIR* (Katsuyama et al., 1994), was "silenced" using siRNAs. Under stringently controlled conditions, CRE- and GRE-dependent transcription induced by taprostene and iloprost were markedly inhibited (74% to 100%) in cells transfected with PTGIR-1 oligonucleotides. Conversely, the same responses evoked by PGE_2 (mediated by EP_2- and EP_4-receptors) were unaffected indicating that these *PTGIR*-targeting siRNAs produced a selective functional knockdown of the desired molecular target. In contrast, the inhibition by taprostene and 15-deoxy-TIC of chemokine output was unaffected in cells in which *PTGIR* was "silenced". Entirely consistent results were obtained when cAMP was selected as the functional readout. Thus, the PTGIR-1 oligonucleotides did not block the cAMP signal induced by 15-deoxy-TIC, whereas the increases in cAMP levels induced by iloprost and taprostene were inhibited by 64% and 44% respectively. These findings cannot easily be reconciled with a single IP-receptor system. Initially, in considering how best

to interpret these data, it was thought that a large IP-receptor reserve may exist on BEAS-2B cells for 15-deoxy-TIC-induced inhibition of chemokine release such that the degree of gene silencing produced by PTGIR-1 siRNAs was insufficient to impair function. However, this possibility was discounted because silencing *PTGIR* will reduce IP-receptor number such that PTGIR-1 should behave in the same way as an inactivating alkylating agent (Kenakin, 1987). Thus, if the known IP-receptor mediates the effect of taprostene and 15-deoxy-TIC on chemokine output, then the *E*/[A] curves that describe these effects should have been displaced to the right in PTGIR-1-transfected cells and, if receptor number is limiting, be associated with a reduction in the maximal asymptote. However, as shown in Fig. 7*D* - *G*, the PTGIR-1 oligonucleotides had no effect on the taprostene or 15-deoxy-TIC *E*/[A] curves as assessed by $p[A]_{50}$ values and maximum inhibition produced. Thus, these latter finding are consistent, again, with the expression of a hitherto undefined IP-receptor ("IP_2") on BEAS-2B cells.

We submit that the *PTGIR* silencing studies also indicate that 15-deoxy-TIC cannot be acting through a splice variant of the known IP-subtype. Indeed, the PTGIR-1 oligonucleotides target sequence in the 5'-UTR of *PTGIR*, and would "silence" all reported putative splice variants. The likelihood that 15-deoxy-TIC suppressed chemokine output by interacting with a heterodimer composed of the IP_1-subtype and an unknown GPCR was also excluded because inactivation of *PTGIR* would reduce the number of dimers and, therefore, the responses they mediate.

In conclusion, the present study provides pharmacological evidence for IP-receptor heterogeneity in BEAS-2B cells. Two functionally-distinct receptors have been characterized that couple to the activation of canonical cAMP/PKA cascades and, independently, regulate reporter (CRE and GRE) activation and inhibition of CXCL9 and CXCL10 release. These receptors can be distinguished with iloprost (IP_1-receptor agonist), 15-deoxy-TIC ("IP_2"-receptor agonist), the IP-receptor antagonist, RO3244794 (affinity at "IP_2" > IP_1), as well by silencing *PTGIR*. We submit that these data cannot

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readily be explained by mRNA splicing or GPCR heterodimerization. Thus, we propose that the RO3244794-sensitive, 15-deoxy-TIC-induced responses in BEAS-2B cells are mediated by the activation of a second receptor for PGI_2 that is distinct from the known IP-subtype encoded by *PTGIR*.

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Authorship Contributions

Participated in research design: Newton, Giembycz

Conducted experiments: Hill

Contributed new reagents: Sheddan

Performed data analysis: Hill, Giembycz

Wrote or contributed to the writing of the manuscript: Newton, Giembycz

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Footnotes

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Figure Legends

Fig 1. Chemical structures of PGI_2, iloprost, taprostene and 15-deoxy-TIC. * denotes chiral center.

Fig. 2. Functional effects of IP-receptor agonists. In *A-H*, BEAS-2B cells were pre-treated (30 min) with vehicle or a combination of BWA 868C (1 μM), L-798,106 (100 nM) and L-161,982 (500 nM). Taprostene (*A* & *E*), PGI_2 (*B* & *F*), iloprost (*C* & *G*), and 15-deoxy-TIC (*D* & *H*) were then added for an additional 30 min followed by IFNγ (100 ng/ml; $p[A]_{85}$). At 24h, the supernatant was harvested and the amount of CXCL9 and CXCL10 was quantified by ELISA. In *I-L*, CRE BEAS-2B reporter cells were pre-treated (30 min) with vehicle or a combination of BWA 868C, L-798,106 and L-161,982 and then exposed to taprostene (*I*), PGI_2 (*J*), iloprost (*K*), and 15-deoxy-TIC (*L*). GRE-reporter cells (*M-P*) were treated identically except that the IP-receptor agonists were added concurrently with dexamethasone (Dex; 1 μM). At 6h supernatants were harvested and luciferase activity was determined. *E*/[A] curves were then constructed from which $p[A]_{50}$ and E_{max} values were determined. Each data point represents the mean ± s.e. mean of *N* independent determinations. See Table 2 for quantification of these data.

Fig. 3. Iloprost and 15-deoxy-TIC do not interact with the same receptor on BEAS-2B cells. In *A* and *B*, *E*/[A] curves were constructed to 15-deoxy-TIC for the inhibition of IFNγ-induced CXCL9 and CXCL10 release respectively in cells pre-treated (30 min) with an antagonist cocktail consisting of BWA 868C (1 μM), L-161,982 (500 nM) and L-798,106 (100 nM) and the antagonist cocktail supplemented with iloprost (1 μM). In *C* & *D*, CRE and GRE reporter cells were pre-treated (30 min) with antagonist cocktail, and iloprost *E*/[A] curves for luciferase expression were then constructed in the absence and presence of 15-deoxy-TIC (1 μM). Each bar and data point represents the mean ± s.e mean of *N* determinations. See text and legend to figure 2 for further details.

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Fig. 4. Determination of the affinity of RO3244794 for antagonizing taprostene-, iloprost and 15-deoxy-TIC-induced responses. BEAS-2B cells were pre-treated (30 min) with RO3244794 (10 pM to 1 μM). Taprostene (*A* & *C*) or 15-deoxy-TIC (*B* & *D*; both 1 μM) was then added followed by IFNγ (100 ng/ml). In parallel, a different culture of cells was pre-treated (30 min) with taprostene (30 pM to 10 μM) and 15-deoxy-TIC (10 pM to 1 μM) in the absence of RO3244794 before being exposed to IFNγ. After 24 the amount of CXCL9 (*A* & *B*) and CXCL10 (*C* & *D*) released into the culture supernatant was quantified by a sandwich ELISA. In *E* & *F*, CRE BEAS-2B reporter cells were pre-treated (30 min) with RO3244794 and then exposed to taprostene (1 nM to 10 μM) or iloprost (100 pM nM to 1 μM. GRE-reporter cells (*G* & *H*) were treated identically except that the IP-receptor agonists agonists were added concurrently with dexamethasone (1 μM). Each pair of *E*/[A] curves was then analyzed simultaneously using a modification (Waud et al., 1978) of the Hill and Gaddum/Schild equations (Lazareno and Birdsall, 1993) as described previously (Ayer et al., 2008) from which pK_b values were derived. Each data point represents the mean ± s.e mean of *N* paired experiments respectively.

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Fig. 5. Effect of taprostene, iloprost and 15-deoxy-TIC on cAMP accumulation in IP*R*-HEK-293 and BEAS-2B cells. In *A* and *B*, IP*R*-HEK-293 and BEAS-2B cells were both pre-treated (30 min) with the PDE inhibitors, rolipram (10 μM; R) and siguazodan (10 μM; S), and then exposed to taprostene, iloprost and 15-deoxy-TIC at the concentrations shown. In *C* and *D*, fixed concentrations of iloprost (Ilo; 100 nM), 15-deoxy-TIC (TIC, 100 nM) and taprostene (Tap, 1 μM) were examined alone and in combination on cAMP levels in BEAS-2B cells. These experiments were performed in the presence of PDEI, BWA 868C (1 μM), L-798,106 (100 nM) and L-161,982 (500 nM) and, where indicated, the IP-receptor antagonist, RO3244794 (RO; 1 μM). Cells were lysed after 45 min and cAMP was measured by EIA. Each bar and data point represent the mean ± s.e. mean of *N* determinations. *P < 0.05, significant difference – one-way ANOVA/Bonnferoni's multiple multiple comparison test.

Fig. 6. Effect of PKIα on iloprost- and 15-deoxy-TIC-induced responses. BEAS-2B cells were infected with Ad5.CMV.PKIα, Ad5.CMV.Null (both MOI = 40) or left untreated (control) for 48h. 15-Deoxy-TIC-induced inhibition of chemokine release (*A* & *B*) and iloprost-induced reporter activation (*C* & *D*) were then measured at 24h and 6h respectively. Data points in each panel represent the mean ± s.e. mean of *N* independent determinations.

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Fig. 7. Effect of silencing *PTGIR* on taprostene, iloprost and 15-deoxy-TIC-induced responses. BEAS-2B cells were transfected with siRNAs (10 nM) directed against *PTGIR* (PTGIR-1) and a universal control (UC). Panel *A* shows the effect of PTGIR-1 and relevant controls on the expression of the variously glycosylated IP-receptor as measured by western blotting. The effects of iloprost (Ilo; 100 nM) and taprostene (Tap; 1 μM) on reporter activation are shown in *B* & *C*. In *D* - *G*, 15-deoxy-TIC and taprostene *E*/[A] curves are shown in untreated cells and in cells transfected with PTGIR-1 and the UC. Each bar and data point represents the mean ± s.e. mean of *N* independent determinations. *P < 0.05, significant inhibition of transcriptional responses in PTGIR-1-transfected cells; One-way ANOVA/ Bonnferoni's multiple comparison test. NS, No Stimulus; Dex, Dexamethasone (1 μM). Key to panel (*A*): **1** Control cells; **2** cells + UC; **3** cells + PTGIR-1; **4** cells + RNAiMax; **5** IP*R*-HEKs (positive control).

Fig. 8. Effect of silencing *PTGIR* on taprostene, iloprost, 15-deoxy-TIC- and PGE_2-induced cAMP accumulation. BEAS-2B cells were transfected with siRNAs directed against *PTGIR* (PTGIR-1), GFP and lamin A/C (Lamin), as well as universal negative control (UC). The transfection lipid RNAiMax was also tested in parallel. Cells were pre-treated with the PDE inhibitors rolipram (10 μM; R) and siguazodan (10 μM; S), BWA 868C (1 μM), L-798,106 (100 nM) and L-161,982 (500 nM), and cAMP accumulation induced by iloprost (Ilo, 100 nM, *A*), taprostene (Tap, 1 μM, *B*) and 15-deoxy-TIC (TIC, 100 nM, *C*). The effect of PGE_2 (1 μM, *D*) was also examined but in the absence of BWA 868C (1 μM), L-798,106 (100 nM) and L-161,982 (500 nM). Each bar represents the mean ± s.e. mean of *N* independent determinations. *P < 0.05, significant inhibition of cAMP accumulation in PTGIR-1-transfected cells. One-way ANOVA/Bonnferoni's multiple comparison test.

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TABLE 1

siRNA Oligonucleotide Sequences

Gene	*Oligonucleotides*	*Accession #*	*Source*
PTGIR[*]	5′-CATCCATCTCATTGTCTAAtt-3′ 3′-ggGTAGGTAGAGTAACAGATT-5′	NM_000960.3	Qiagen
PTGIR[†]	5′-GGGCGACAGGAGCCAGAAAtt-3′ 3′-agCCCGCTGTCCTCGGTCTTT-5′	NM_000960.3	Qiagen
GFP	5′-GGCAAGCTGACCCTGAAGTTCtt-3′ 3′-ttCCGTTCGACTGGGACTTCAAG-5′	U57609	Dharmacon
Lamin A/C	5′-CTGGACTTCCAGAAGAACAtt-3′ 3′-ttGACCTGAAGGTCTTCTTGT-5′	NM_005572	Qiagen
GAPDH	5′-GAGCCACATCGCTCAGACAtt-3′ 3′-ggCTCGGTGTAGCGAGTCTGT-5′	NM_002046	Qiagen

In the text, * and [†] refer to PTGIR-1 and PTGIR-2 siRNA sequences respectively.

TABLE 2

Relative potencies and functional activity of IP-receptor agonists in BEAS-2B Cells

	Functional Output							
	Inhibition of CXCL9 Release		*Inhibition of CXCL10 Release*		*Induction of CRE-Reporter*		*Augmentation of GRE-Reporter*	
	$p[A]_{50}$	*Max (%)*	$p[A]_{50}$	*Max (%)*	$p[A]_{50}$	*Max (fold)*	$p[A]_{50}$	*Δ (fold)*[b]
Iloprost	-9.08 ± 0.07 (6)[c]	20.9 ± 2.6 (6)[c]	-8.93 ± 0.06 (7)[c]	24.7 ± 2.5 (6)[c]	-8.12 ± 0.04 (6)	3.60 ± 0.22 (6)	-8.00 ± 0.04 (3)	26.1 ± 1.3 (3)
Iloprost + Antagonists[a]	Inactive at 1 μM		Inactive at 1 μM		-8.09 ± 0.04 (6)	3.80 ± 0.23 (6)	-8.01 ± 0.04 (3)	26.8 ± 1.4 (3)
Taprostene	-7.30 ± 0.05 (7)	35.5 ± 1.8 (7)	-7.26 ± 0.07 (7)	35.7 ± 1.9 (7)	-7.19 ± 0.06 (6)	3.55 ± 0.10 (6)	-6.53 ± 0.21 (3)	34.3 ± 2.1 (3)
Taprostene + Antagonists[a]	-7.40 ± 0.17 (7)	33.4 ± 1.6 (7)	-7.26 ± 0.11 (7)	33.4 ± 2.5 (7)	-7.18 ± 0.05 (6)	3.52 ± 0.18 (6)	-6.63 ± 0.05 (3)	35.3 ± 2.0 (3)
15-Deoxy-TIC	-7.76 ± 0.03 (3)	43.1 ± 1.7 (3)	-7.79 ± 0.01 (3)	42.3 ± 0.5 (3)	Inactive at 1 μM		Inactive at 1 μM	
15-Deoxy-TIC + Antagonists[a]	-7.70 ± 0.09 (3)	45.4 ± 1.8 (3)	-7.81 ± 0.04 (3)	42.0 ± 0.4 (3)	Inactive at 1 μM		Inactive at 1 μM	
PGI_2	-5.96 ± 0.07 (3)	49.1 ± 1.2 (3)	-5.99 ± 0.08 (3)	53.0 ± 1.7 (3)	*e*	6.3 ± 0.1 (4)[d]	*e*	8.0 ± 1.0 (3)[d]
PGI_2 + Antagonists[a]	-5.93 ± 0.09 (3)	48.7 ± 1.9 (3)	-5.97 ± 0.09 (3)	52.4 ± 2.5 (3)	*e*	6.0 ± 0.2 (4)[d]	*e*	7.6 ± 0.9 (3)[d]

a Experiment performed in cells pre-treated with BWA 868C (1 μM), L-798,106 (100 nM) and L-161,982 (500 nM)

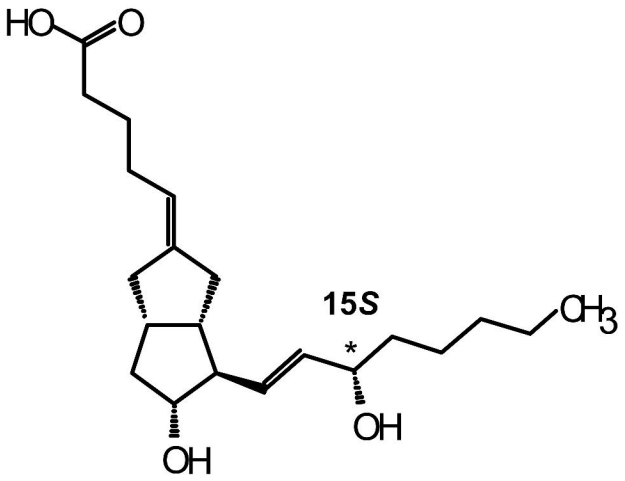
b Fold induction: (Dexamethasone + Agonist) – Dexamethasone alone

c Response biphasic; $p[A]_{50}$ and Max values refer to high potency component

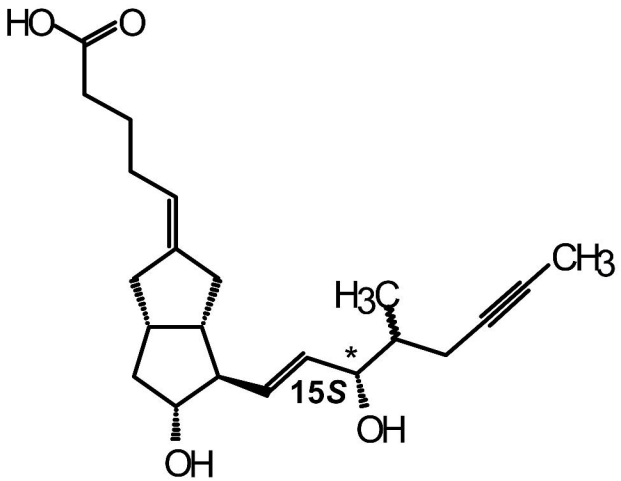
d Fold/Δfold induction @ 100 μM

e $p[A]_{50}$ value could not be determined because maximum response not attained

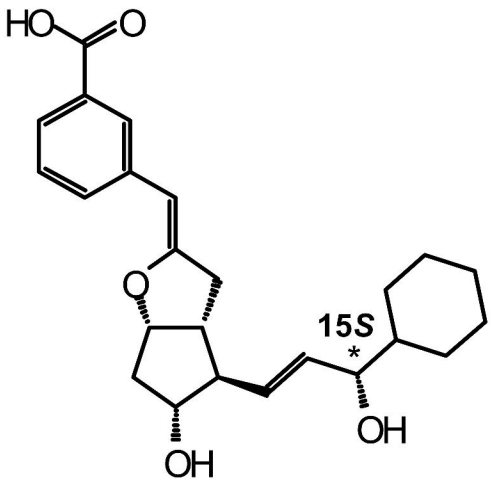
Figure 1



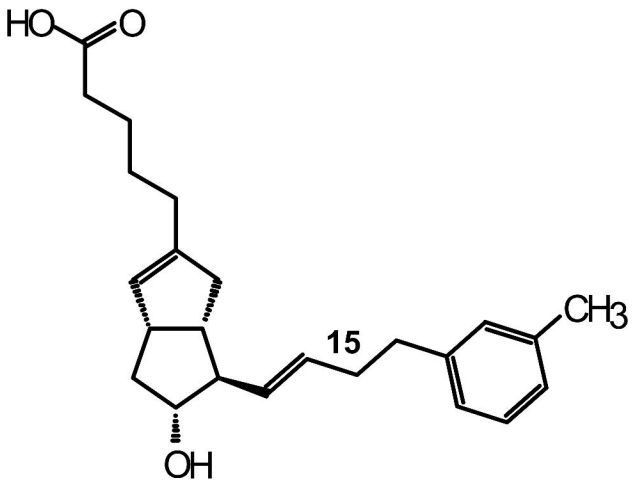
Prostacyclin



Iloprost



Taprostene



15-Deoxy-TIC

Figure 2

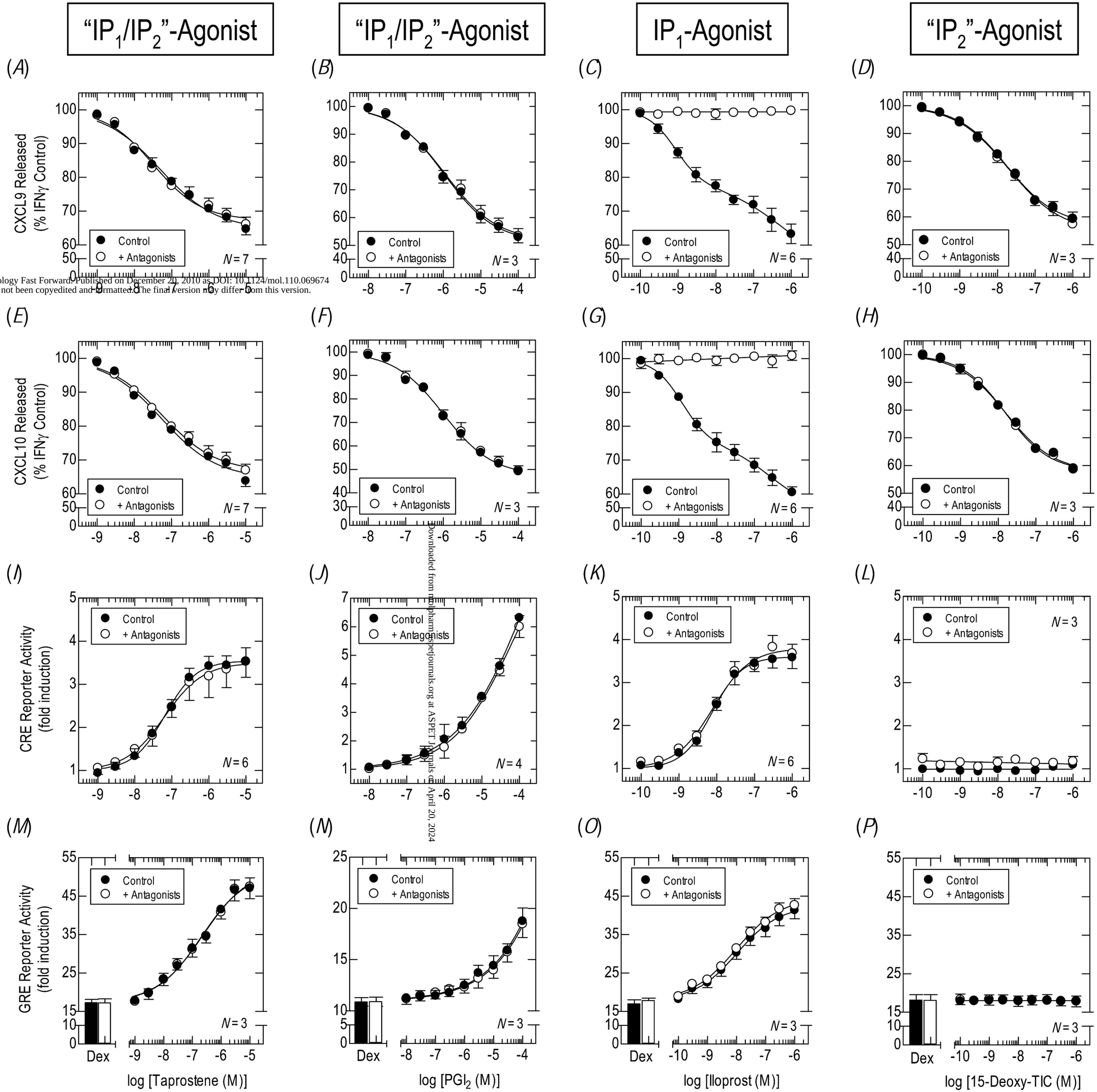


Figure 3

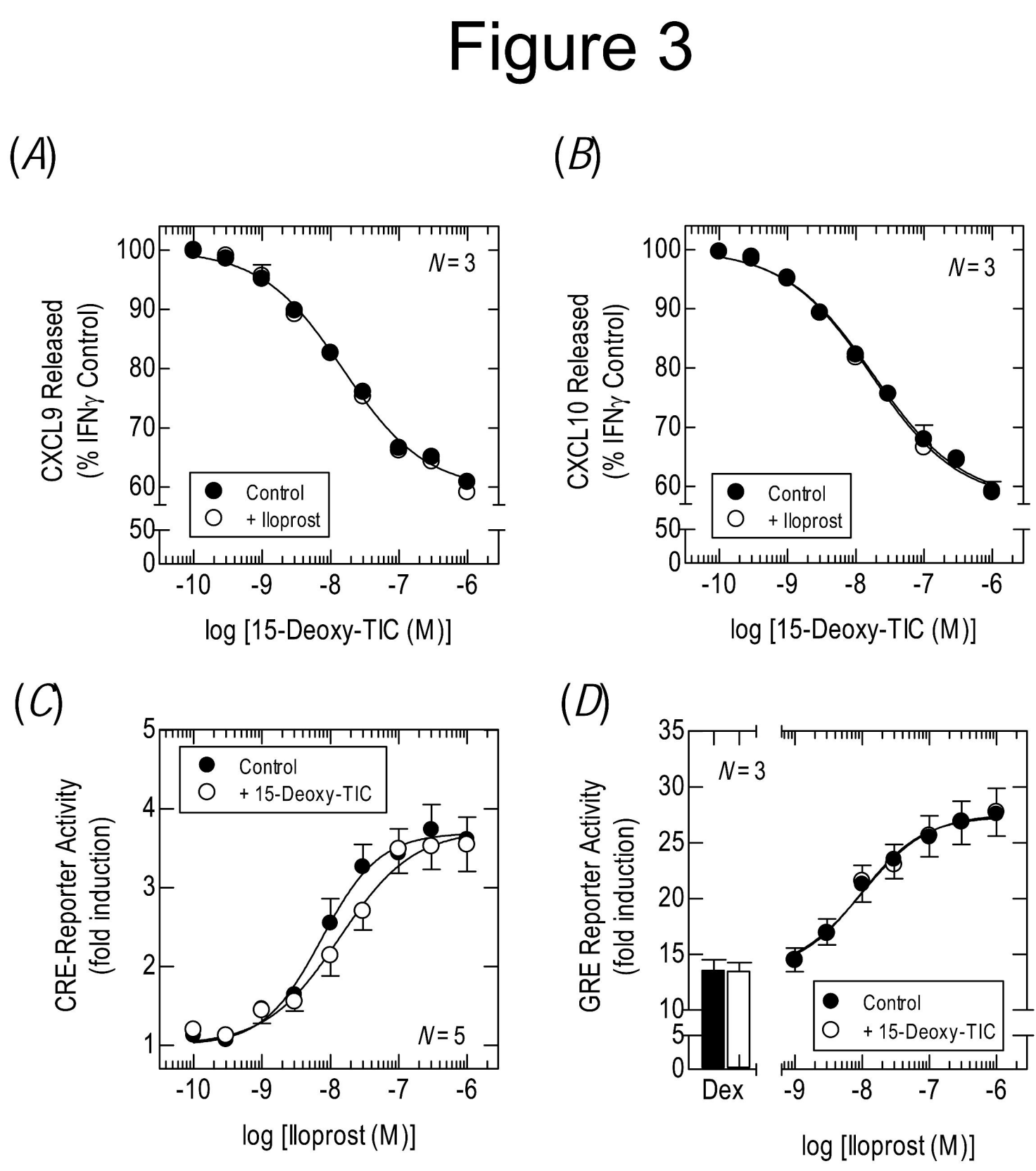

(A)
CXCL9 Released
(% IFNγ Control)
100
90
80
70
60
50
0
N = 3
Control
+ Iloprost
-10
-9
-8
-7
-6
log [15-Deoxy-TIC (M)]
(B)
CXCL10 Released
(% IFNγ Control)
100
90
80
70
60
50
0
N = 3
Control
+ Iloprost
-10
-9
-8
-7
-6
log [15-Deoxy-TIC (M)]
(C)
CRE-Reporter Activity
(fold induction)
5
4
3
2
1
Control
+ 15-Deoxy-TIC
N = 5
-10
-9
-8
-7
-6
log [Iloprost (M)]
(D)
GRE Reporter Activity
(fold induction)
35
30
25
20
15
10
5
0
N = 3
Dex
Control
+ 15-Deoxy-TIC
-9
-8
-7
-6
log [Iloprost (M)]

Figure 4

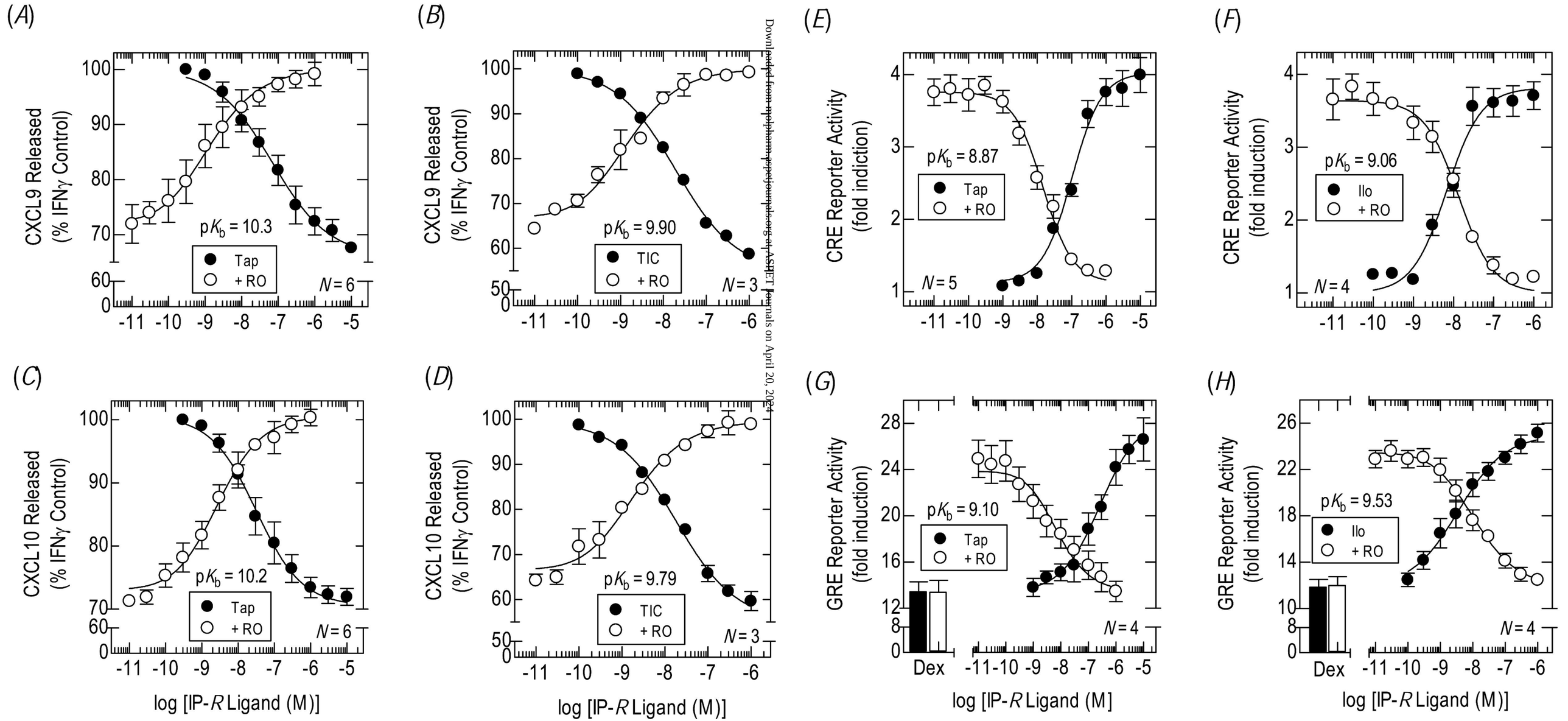


Figure 5

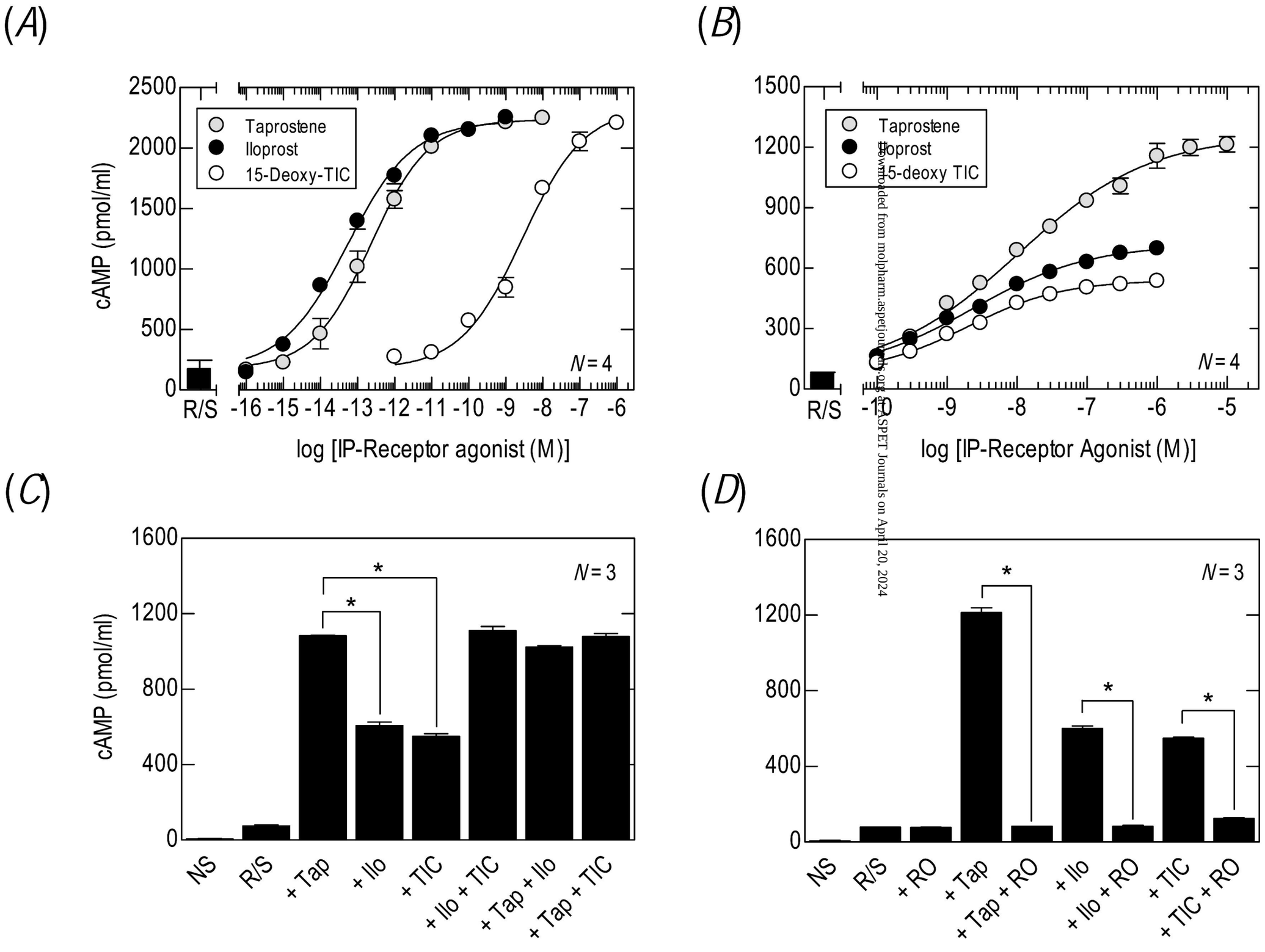


Figure 6

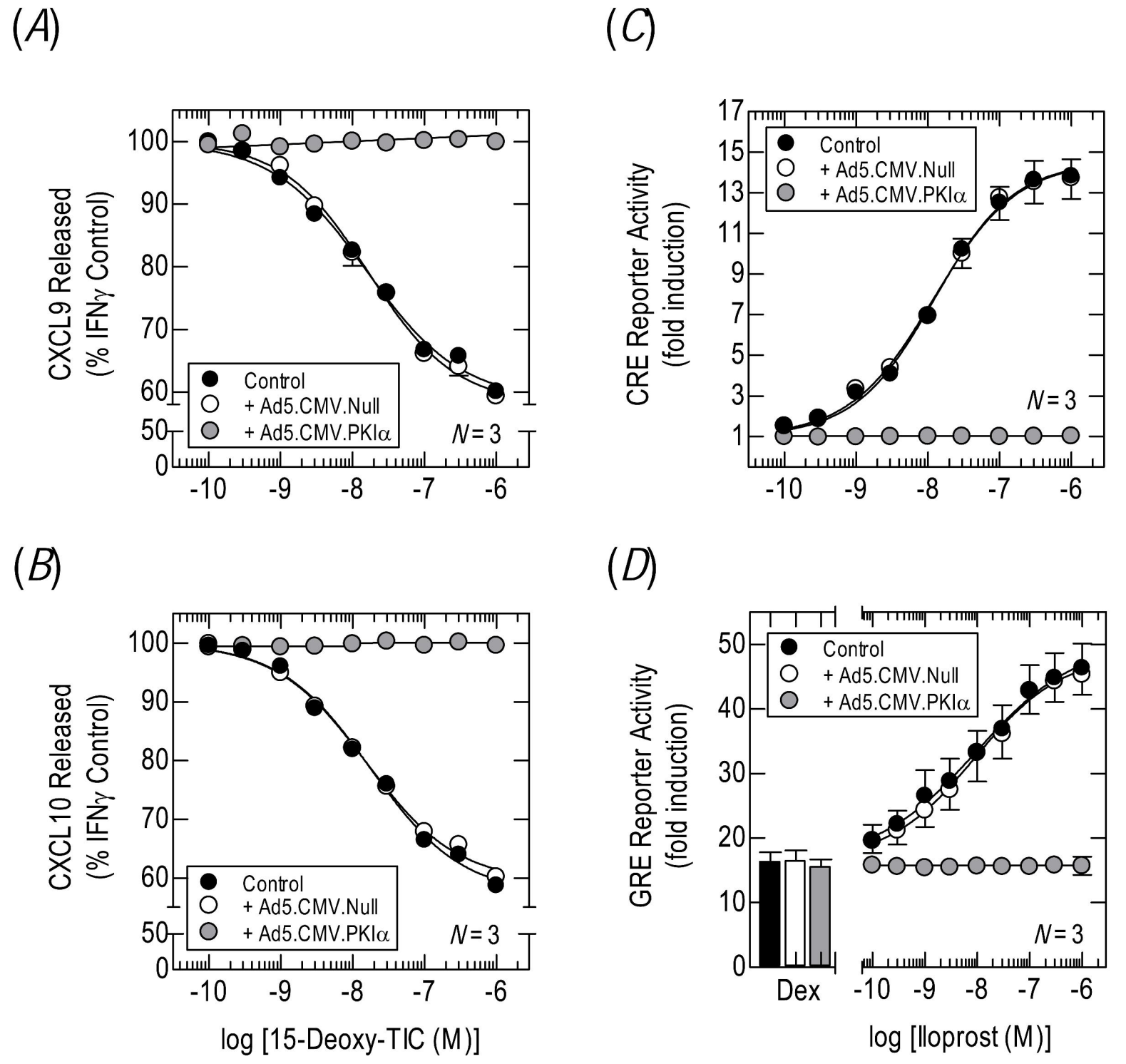
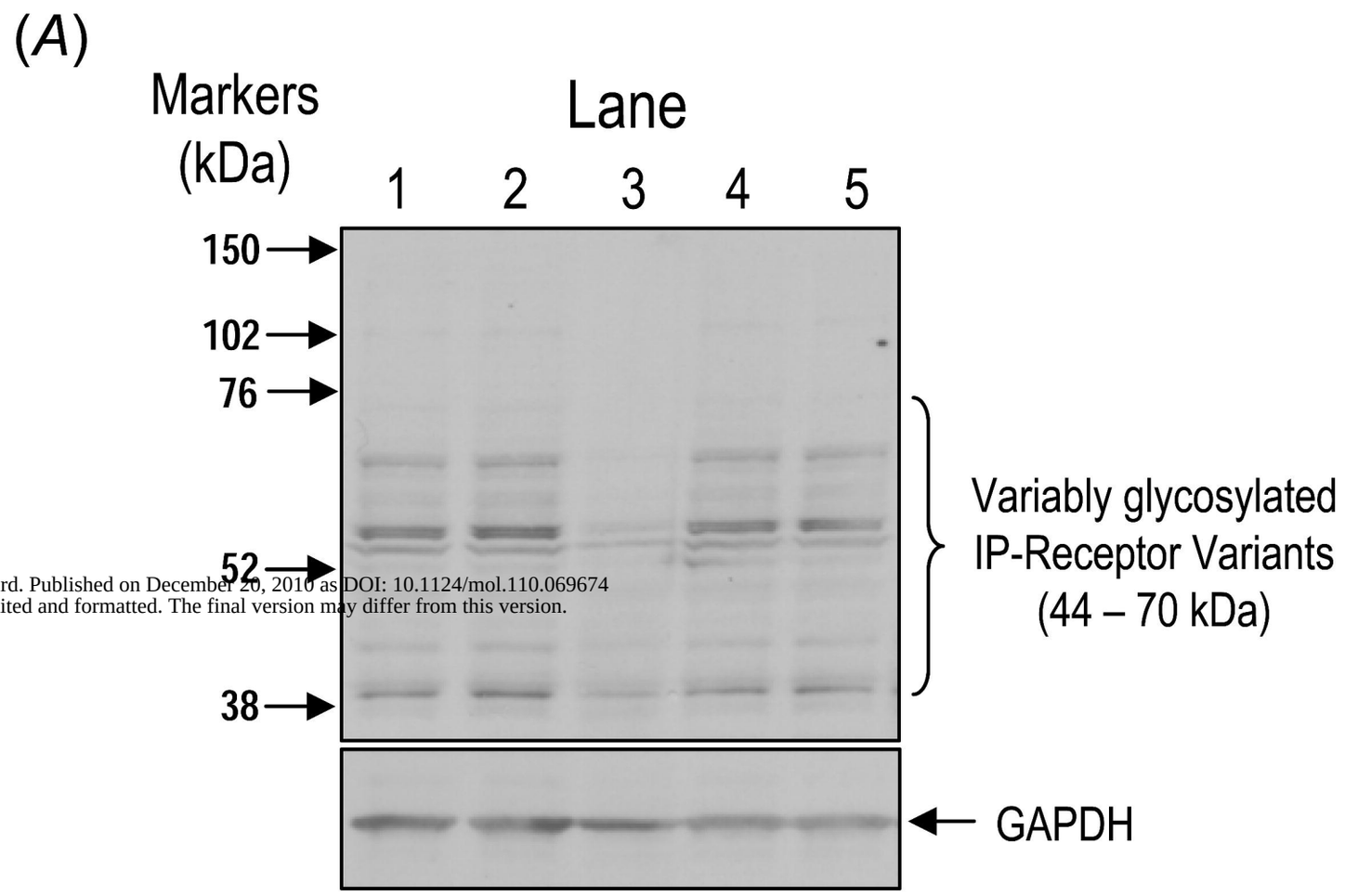
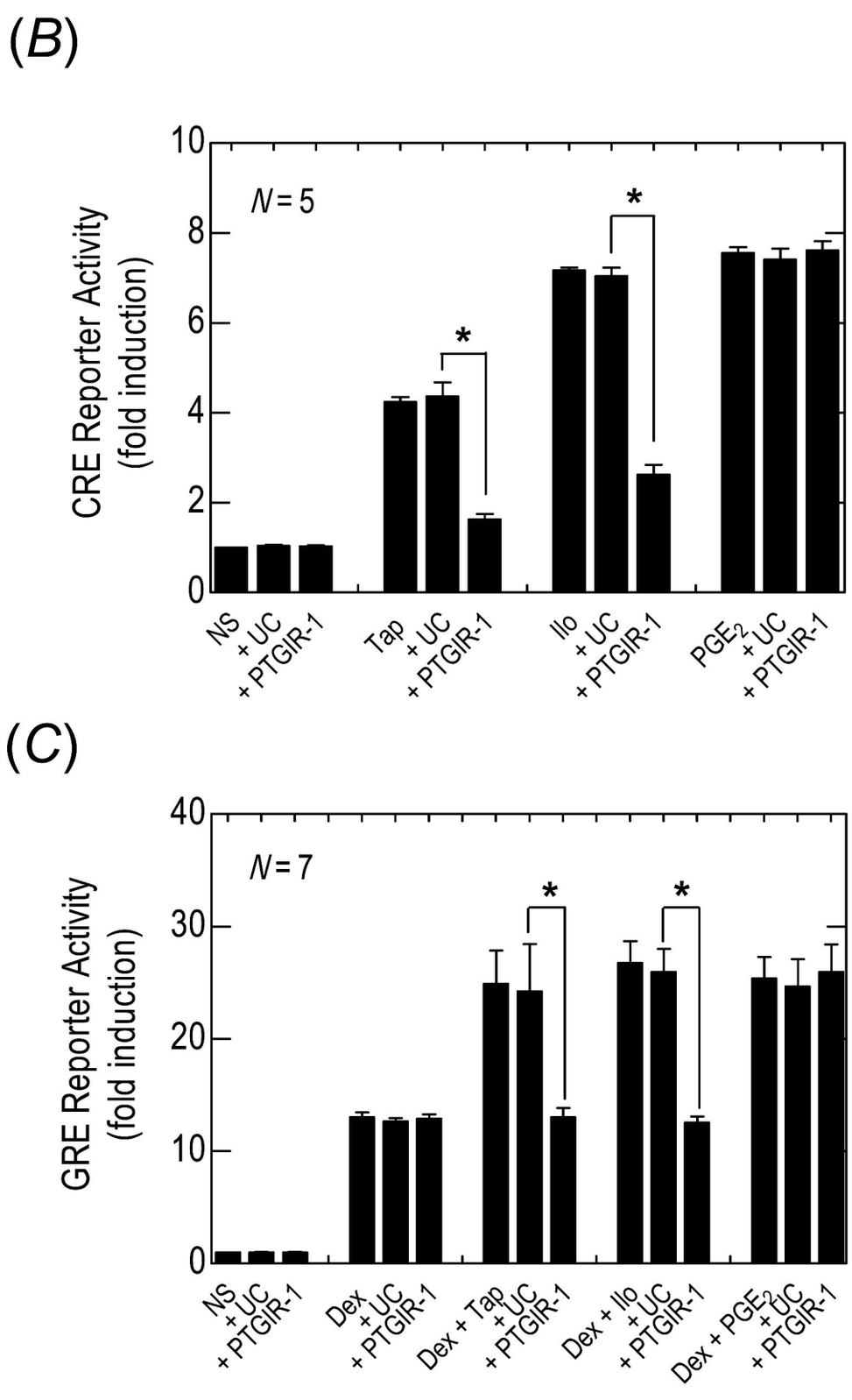
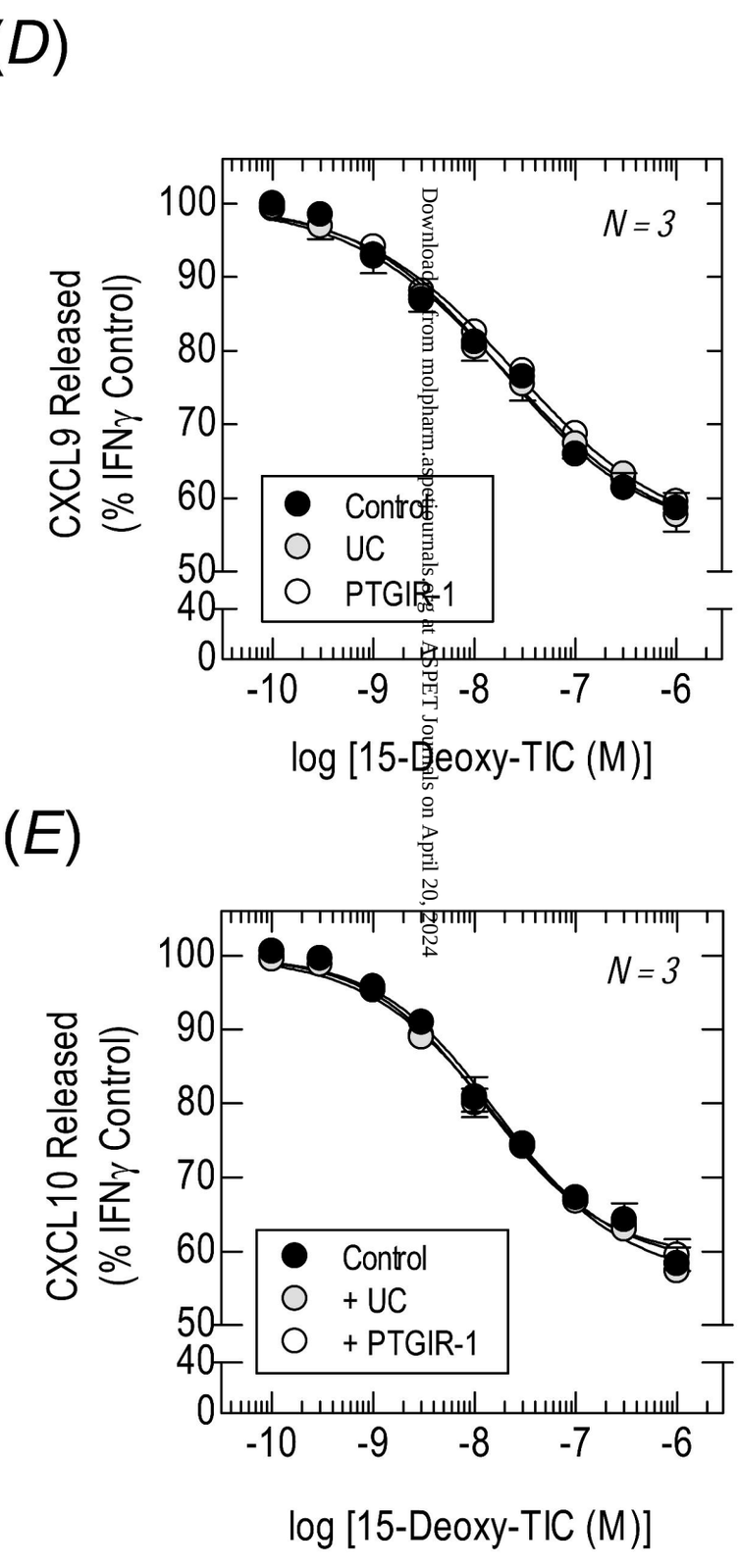

(A)
CXCL9 Released (% IFNγ Control)
100
90
80
70
60
50
0
-10
-9
-8
-7
-6
Control
+ Ad5.CMV.Null
+ Ad5.CMV.PKIα
N = 3
(B)
CXCL10 Released (% IFNγ Control)
log [15-Deoxy-TIC (M)]
(C)
CRE Reporter Activity (fold induction)
17
15
13
11
9
7
5
3
1
(D)
GRE Reporter Activity (fold induction)
50
40
30
20
10
0
Dex
log [Iloprost (M)]

Figure 7







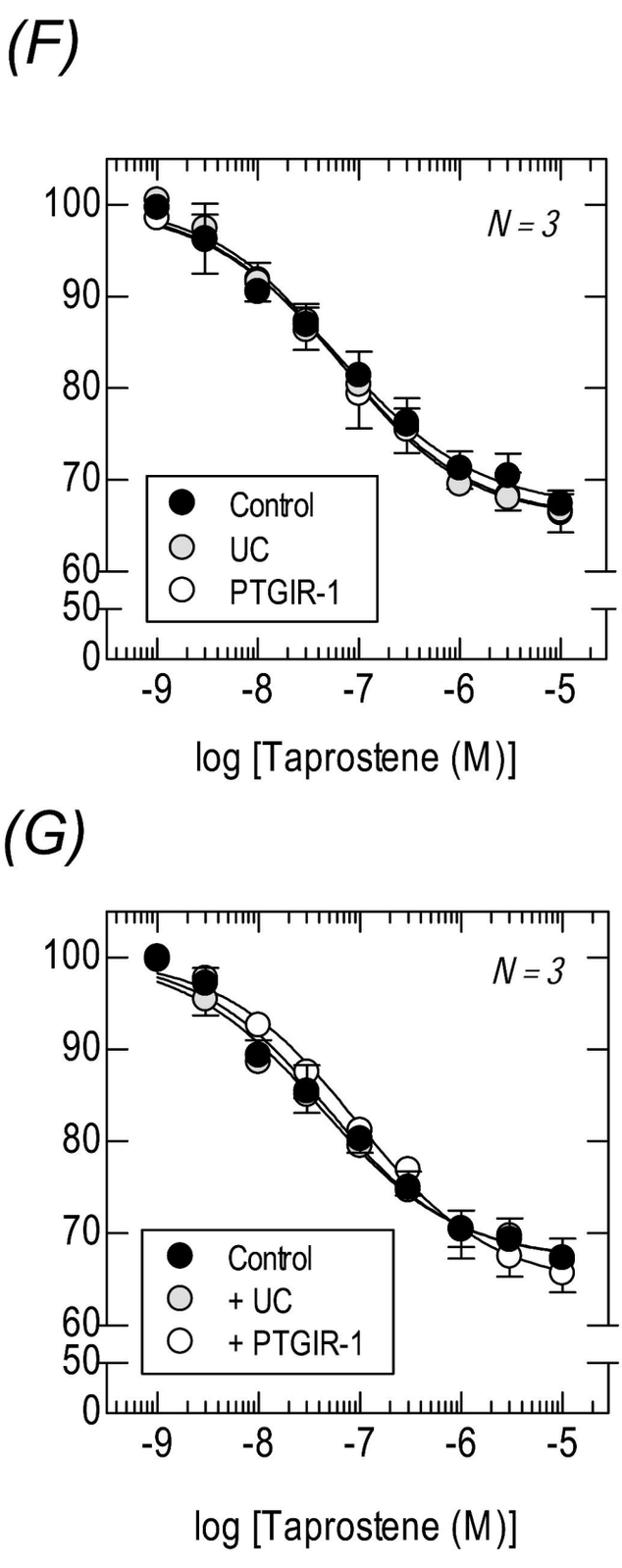
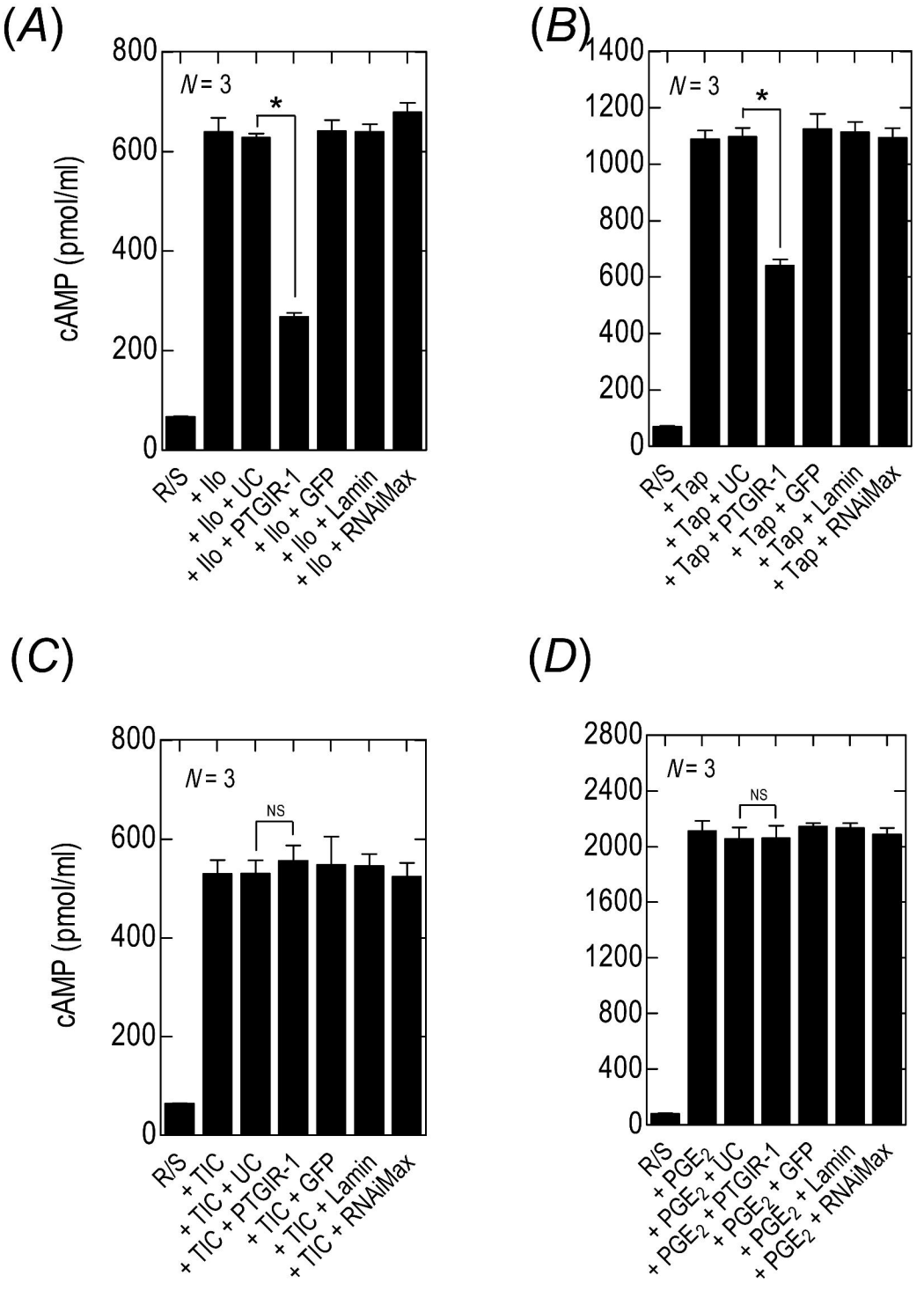


Figure 8


(A)
cAMP (pmol/ml)
N = 3
*
R/S
+ Ilo
+ Ilo + UC
+ Ilo + PTGIR-1
+ Ilo + GFP
+ Ilo + Lamin
+ Ilo + RNAiMax
(B)
N = 3
*
R/S
+ Tap
+ Tap + UC
+ Tap + PTGIR-1
+ Tap + GFP
+ Tap + Lamin
+ Tap + RNAiMax
(C)
cAMP (pmol/ml)
N = 3
NS
R/S
+ TIC
+ TIC + UC
+ TIC + PTGIR-1
+ TIC + GFP
+ TIC + Lamin
+ TIC + RNAiMax
(D)
N = 3
NS
R/S
+ PGE2
+ PGE2 + UC
+ PGE2 + PTGIR-1
+ PGE2 + GFP
+ PGE2 + Lamin
+ PGE2 + RNAiMax