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The Xenoestrogens Biphenol-A and Nonylphenol differentially regulate metalloprotease-mediated shedding of EGFR ligands

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Abstract

The xenoestrogens Bisphenol-A (BPA) and Nonylphenol (NP) are endocrine disruptors used in the plastic polymer industry to manufacture different products for human use. Previous studies have suggested a role of these compounds in the shedding of signaling molecules, such as tumor necrosis factor α (TNF- α). The aim of this work was to evaluate the effect of BPA and NP on the sheddase ADAM17 and its newly discovered regulators iRhom1 and iRhom2 in the release of EGFR-ligands. We report that BPA and NP can stimulate the release of the ADAM17-substrates HB-EGF and TGF- α . In cells lacking ADAM17 (*Adam17*^{-/-} mEFs) BPA-stimulated release of HB-EGF, but not TGF- α , was strongly reduced, whereas NP-stimulated shedding of HB-EGF and TGF- α was completely abolished. Inactivation of both ADAM17 and the related ADAM10 (*Adam10/17*^{-/-} mEFs) completely prevented the release of these substrates. In the absence of iRhom1, BPA- or NP-stimulated release of HB-EGF or TGF- α was comparable to wild type control mEFs, conversely the BPA-induced release of HB-EGF was abolished in *iRhom2*^{-/-} mEFs. The defect in shedding of HB-EGF in *iRhom2*^{-/-} mEF cells could be rescued by overexpressing iRhom2. Interestingly, the NP-stimulated release of HB-EGF was not affected by the absence of

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iRhom2, suggesting that NP could potentially activate both ADAM10 and ADAM17. We tested this hypothesis using betacellulin (BTC), an EGFR-ligand that is a substrate for ADAM10. We found that NP, but not BPA stimulated the release of BTC in *Adam17*^{-/-}, *iRhom2*^{-/-} or *iRhom1/2*^{-/-}, but not in *Adam10/17*^{-/-} cells. Taken together, our results suggest that BPA and NP stimulate the release of EGFR-ligands by differentially activating ADAM17 or ADAM10. The identification of specific effects of these endocrine disruptors on ADAM10 and ADAM17 will help to provide a better understanding of their roles in cell signaling and proinflammatory processes, and provide new potential targets for treatment of reproductive or inflammatory diseases such as asthma or breast cancer that are promoted by xenoestrogens.

Keywords

ADAM; xenobiotic; epidermal growth factor receptor (EGFR); growth factor

1. INTRODUCTION

Endocrine disruptors (ED) is a term that refers to a wide variety of compounds that disrupt the internal hormonal balance in animals and humans, leading to transient and/or permanent effects (Lagos-Cabre and Moreno, 2012). Major concerns have recently been raised because compounds considered EDs such as Bisphenol A (BPA) and Nonylphenol (NP) are commonly used in the plastic polymer industry (Lagos-Cabre and Moreno, 2012). NP is a degradation product of alkylphenol ethoxylates, which is used for manufacturing plastic compounds for dentistry, food packaging, textiles, pesticides, detergents, paints, and cosmetics (E.P.A., 2010). Several animal studies have shown severe adverse effects of NP in the male reproductive tract, such as reduced testis size, germ cell apoptosis and decreased production of spermatozoa (Hossaini et al., 2001; McClusky et al., 2007; Nagao et al., 2001; Urriola-Munoz et al., 2014).

The monomer BPA is a weak estrogenic chemical that is used in the manufacturing of polycarbonate plastics and epoxy resins. BPA can leech from polycarbonate products when incomplete polymerization occurs with the epoxy resin, reaching food and liquids, which are subsequently consumed by humans (Wetherill et al., 2007). Many reports have shown that BPA has a proliferative effect on reproductive tissues such as mammary glands or the uterus in animal models (Vandenberg et al., 2009; Vandenberg et al., 2007). In addition to the effect of BPA on the endocrine system, it can also disrupt the immune system by interfering with the production as well as the release of cytokines involved in immune responses (Rogers et al., 2013). In this regard, *in vitro* experiments show that BPA stimulates the release of TNF- α from Sertoli cells primary culture (Urriola-Muñoz et al., 2014), murine microglial cells (Zhu et al., 2015), human adipose tissue explants (Ben-Jonathan et al., 2009), mouse bone marrow-derived mast cell (O'Brien et al., 2014) and human cytotrophoblasts (Benachour and Aris, 2009).

TNF- α is synthesized as a membrane-anchored precursor that is released from cells by the transmembrane metalloprotease named ADAM17 (a disintegrin and metalloprotease 17), also known as TACE (TNF- α Converting Enzyme) (Black et al., 1997; Blobel, 2005; Moss et

al., 1997). ADAM17 is also responsible for the “shedding” or proteolytic release of different membrane-bound receptors and ligands such as Transforming Growth Factor- α (TGF- α), Heparin-Binding Epidermal Growth Factor (HB-EGF), and many others (Hinkle et al., 2004; Murphy, 2008; Sahin et al., 2004). The inactive Rhomboid iRhom2, a catalytically inactive member of the Rhomboid family of intramembrane proteinases (Adrain et al., 2012; McIlwain et al., 2012; Siggs et al., 2012), was recently identified as a vital regulator of the function and substrate selectivity of ADAM17 (Adrain et al., 2012; Brooke et al., 2014; Maretzky et al., 2013; McIlwain et al., 2012; Siggs et al., 2012). Moreover, mice lacking iRhom1 and iRhom2 resemble mice lacking ADAM17, suggesting that a principal function of iRhoms1 and 2 is to regulate the function of ADAM17 (Li et al., 2015). The main goal of this study was to determine whether ADAM17 and iRhoms 1 or 2 are required for the stimulated shedding of TGF- α and HB-EGF by the xenoestrogens BPA and NP.

2. EXPERIMENTAL PROCEDURES

2.1 Chemicals

Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] (BPA), 4-nonylphenol (NP), phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore, ionomycin, were obtained from Sigma (MO, USA). The ADAM10 and ADAM17 inhibitors GW280264X and GI254023X, were obtained from AOBIOUS INC (MA, USA). The metalloproteinase inhibitor marimastat (BB2516) was purchased from Tocris Biosciences (Bristol, UK).

2.2 Expression Vectors

The expression vector for iRhom2 and plasmids encoding alkaline phosphatase (AP)-tagged EGFR ligands have been described previously (Maretzky et al., 2013; McIlwain et al., 2012; Sahin et al., 2004). A plasmid for mitotic arrest deficient 2 (MAD2) was used as a negative control (Nelson et al., 1999).

Cell lines and Cell Transfection and Shedding Assays

Embryonic fibroblasts were isolated from E13.5 *iR1*^{-/-} and *iR1/2*^{-/-} embryos to generate primary mEFs for immortalization as previously described (Sahin et al., 2006). Briefly, the head and viscera of E13.5 embryos were removed and the remaining tissue was minced and then trypsinized in 0.25% trypsin for 15 minutes at 37°C. Cells were released through mechanical trituration and grown in Dulbecco's modified Eagle medium supplemented with antibiotics and 10% fetal calf serum. Primary mEF cells were immortalized by transfection with a plasmid carrying the simian virus 40 (SV40) large T (Sahin et al., 2004).

Mouse embryonic fibroblasts (mEFs) lacking ADAM10, ADAM17 or ADAM10 and ADAM17 (Le Gall et al., 2009), or lacking iRhom1, iRhom2 or iRhom1 and iRhom2 (Li et al., 2015) were co-transfected with plasmids encoding alkaline phosphatase (AP)-tagged EGFR ligands and expression vectors for iRhom2 or the mitotic arrest deficient 2 (MAD2) as a control. Briefly, these immortalized mEFs were grown to 80% confluency and then were washed and cultured in Opti-MEM medium deprived of serum, antibiotic and antimycotic for 1 h. After that, the cells were cultured for 6 h with DNA-LipofectAMINE complexes with the vectors listed above, and finally the cells were cultured for 24 h in

DMEM-F12 medium with 10% FBS and 10% antibiotic and antimycotic. One day after transfection, the cells were washed in Opti-MEM (Gibco) for 1 hr. Fresh Opti-MEM with 300 μ M BPA, 50 μ M NP, 25 ng/ml Phorbol 12-myristate 13-acetate (PMA) or 2.5 μ M ionomycin was added to the cells for 1 hour to stimulate shedding, as indicated. The hydroxamate inhibitors GW280264X or GI254023X were pre-incubated with cells for 30 minutes at a concentration of 1 μ M where indicated. PMA was used as a positive control for the induced shedding of TGF- α and HB-EGF, on the other hand, ionomycin (IM) was used as a positive control of the induced shedding of BTC. PMA and IM are a commonly used activator of ectodomain shedding (Hooper et al., 1997; Horiuchi et al., 2007; Massague and Pandiella, 1993; Sahin et al., 2004). Protein ectodomain shedding assays using alkaline phosphatase (AP)-tagged substrates in mEFs were performed as described (Horiuchi et al., 2007; Sahin et al., 2004; Zheng et al., 2004). After incubating with the alkaline phosphatase substrate, 4-nitrophenyl phosphate, the alkaline phosphatase activity in the supernatant and lysate were measured at A405. Three identical wells were prepared, and the ratio of AP activity in the supernatant and that of the cell lysate plus supernatant was calculated. For stimulated shedding or vehicle, the fold increase in the ratio of AP activity obtained after stimulation is shown relative to ratio of AP activity in control wells. Each experiment was conducted at least three times. Transfection efficiency was controlled for by determining the ratio of the activity of AP in the medium over the AP-activity in the cells plus the medium.

2.4 Statistical analysis

Mean values were compared using analysis of variance (ANOVA). When the ANOVA test showed statistical differences, the Tukey post test was used to discriminate between groups. Statistical significance was defined as $p < 0.05$ (Sokal, 1995). Statistical analysis were performed before data normalization with raw data using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Graphs show the mean $\pm$ S.E.M.

3. RESULTS AND DISCUSSION

To test whether xenoestrogens could stimulate the release of EGFR-ligands from cells, we examined whether BPA or NP could activate the release of TGF- α and HB-EGF from wild type immortalized mouse embryonic fibroblasts (mEFs). We found that 300 μ M BPA or 50 μ M NP were the lowest concentrations that stimulated a significant increase in the shedding of TGF- α from mEFs after 1 h of incubation (Fig. 1A, B). These concentrations used in this work would be similar to those found in urine of humans exposed daily to high concentrations of these xenoestrogens, such as plastics factory workers, because BPA concentrations in these exposed workers are up to 500 higher than the concentration in unexposed people (Li et al., 2010). Cell morphology and viability remained intact at these concentrations of BPA or NP (data not shown). At these same concentrations, BPA and NP were also able to stimulate the release of HB-EGF (Fig. 1C, D). BPA was less potent than phorbol-12-myristate-13-acetate (PMA) in stimulating shedding of TGF- α and HB-EGF (Fig. 1A, C), whereas NP stimulated TGF α to a similar extent as PMA (Fig. 1B), and HB-EGF shedding to a greater extent than PMA (Fig. 1D). Next, we tested whether the BPA or NP-stimulated release of TGF- α and HB-EGF depended on ADAM17 using *Adam17*^{-/-}

mEFs, or on both ADAM17 and the related ADAM10, using *Adam17/10*^{-/-} mEFs. We found that the BPA-stimulated release of HB-EGF was almost completely abolished in *Adam17*^{-/-} mEFs (Fig. 2A, E), whereas the BPA-stimulated shedding of TGF-α was only slightly reduced in these cells (Fig. 2B, 2F). However, PMA-stimulated shedding of both substrates was abrogated in *Adam17*^{-/-} mEFs, as previously described (Horiuchi et al., 2007; Le Gall et al., 2009). NP-stimulated shedding of HB-EGF was also abolished in *Adam17*^{-/-} mEFs as opposed to wild type controls (Fig. 2C, G), and shedding of TGF-α was strongly reduced (Fig. 2D, H). These results indicate that BPA and NP-stimulated shedding of HB-EGF as well as NP-stimulated release of TGF-α were dependent on ADAM17, whereas BPA-stimulated release of TGF-α was not. Since the ablation of ADAM17 only partially reduced the BPA-stimulated shedding of TGF-α, it is possible that one or more other proteinases besides ADAM17 could be activated by BPA.

Previous studies have shown that calcium influx can activate ADAM10 as the sheddase for TGF-α and other ADAM17 substrates in *Adam17*^{-/-} mEFs (Horiuchi et al., 2007; Sahin et al., 2004). Since BPA promotes dysregulation of calcium homeostasis (Lee et al., 2008; Yan et al., 2011), we hypothesized that BPA stimulates the release of TGF-α by activating ADAM10. When we performed similar experiments in *Adam10/17*^{-/-} cells, we found that the release of both HB-EGF and of TGF-α was abolished (Fig. 2I and J). This result indicates that the remaining BPA-stimulated shedding of TGF-α in *ADAM17*^{-/-} mEFs depends on ADAM10. The NP-stimulated shedding of HB-EGF and TGF-α was also not detectable in *Adam10/17*^{-/-} cells (Fig. 2K and L).

To further corroborate these findings, we decided to evaluate the shedding of TGF-α in wild type mEFs treated with the hydroxamate-type metalloproteinase inhibitor GI254023X, which is selective for ADAM10 at 1 μM (Le Gall et al., 2009), or GW280264X, which inhibits both ADAM10 and ADAM17 (Hundhausen et al., 2003). Our results showed that BPA, NP and PMA-stimulated shedding of TGF-α was strongly inhibited by GW280264X, but not by GI254023X in wild type mEFs (Fig. 3A). When we performed similar experiments in *Adam17*^{-/-} mEFs, we found that NP-stimulated release of TGF-α was not detectable in these cells, suggesting that this stimulus is ADAM17-dependent. However, we observed a similar induction of TGF-α shedding in *Adam17*^{-/-} mEFs stimulated by BPA as in wild type mEFs, which was prevented by GI254023X and GW280264X (Fig. 3B). The presence of GI254023X or GW280264X did not affect the unstimulated basal shedding of TGF-α in wild type or *Adam17*^{-/-} mEFs. Thus, our results indicate that in wild type cells, release of BPA-stimulated TGF-α depends on ADAM17, and that ADAM10 can only compensate for the absence of ADAM17 in genetically modify *Adam17*^{-/-} mEFs.

Given that BPA and NP stimulate the release of TGF-α and HB-EGF, we tested whether the shedding of these substrates was dependent on the presence of iRhom1 or iRhom2, two recently identified regulators of ADAM17 (Adrain et al., 2012; Issuree et al., 2013; Maretzky et al., 2013; McIlwain et al., 2012; Siggs et al., 2012). First, we evaluated the release of TGF-α and HB-EGF in iRhom1-deficient mEFs. We found that BPA- and NP-induced shedding of TGF-α and HB-EGF in *iRhom1*^{-/-} mEFs was comparable to wild type control mEFs (Fig. 4). Our results confirmed previous studies showing that inactivation of iRhom1 alone does not affect basal or stimulated ADAM17 activity (Maretzky et al., 2013),

and suggest that the BPA- or NP-stimulated release of HB-EGF and TGF- α in mEFs does not require iRhom1.

Next we asked whether BPA or NP could stimulate the shedding of HB-EGF or TGF- α from mEFs lacking iRhom2 (*iRhom2*^{-/-}), and whether any differences could be rescued by overexpressing iRhom2. Previous studies have shown that shedding of HB-EGF depends on iRhom2/ADAM17, whereas shedding of TGF- α requires ADAM17 but can occur in the absence of iRhom2 (Maretzky et al., 2013). We found that transfection of wild type mEFs with either MAD2 control vector or a vector expressing iRhom2 had no significant effect on the release of TGF- α (Fig. 5A and C) or HB-EGF (Fig. 5B and D) stimulated by BPA (Fig. 5A and B) or NP (Fig. 5C and D). Treatment with BPA stimulated the release of TGF- α but not HB-EGF in *iRhom2*^{-/-} mEF transfected with MAD2 control (Fig. 5E and F, white bars). PMA showed the same effect as BPA in these cells. However, when *iRhom2*^{-/-} mEF cells were transfected with iRhom2, the BPA- and PMA- stimulated release of HB-EGF was restored (Fig. 5F, black bars). Thus, the presence of iRhom2 is essential for the release of HB-EGF, but not TGF- α , in mEFs stimulated by BPA. Similarly, the NP-stimulated shedding of TGF- α was independent of the presence of iRhom2 (Fig. 5G). Interestingly, the release of HB-EGF was still stimulated by NP in *iRhom2*^{-/-} mEFs, albeit at substantially lower levels than in wild type mEFs (Fig. 5H, white bars), whereas PMA-dependent stimulation of HB-EGF-shedding was abolished in *iRhom2*^{-/-} mEFs (Fig. 5H).

When we performed similar experiments in wild type cells treated with GW280264X or GI254023X, we found that BPA- and NP-stimulated shedding of HB-EGF was strongly reduced by both inhibitors, whereas PMA stimulated was only significantly reduced by GW280264X (Fig 6A). Interestingly, the remaining NP-dependent shedding of HB-EGF from *iRhom2*^{-/-} mEFs and *iRhom1/2*^{-/-} mEFs was prevented by GW280264X and GI254023X (Fig 6B, C), suggesting that this release was most likely due to residual activity of ADAM10. These results suggest that stimulation with NP can induce shedding of HB-EGF even in the absence of iRhom2 or iRhom1 and 2, albeit at lower levels, and that the responsible proteinase is ADAM10.

To test this hypothesis we used betacellulin (BTC), an EGFR-ligand that is specifically shed by ADAM10 (Sahin et al., 2004; Sanderson et al., 2005). We found that neither BPA nor NP stimulated the release of BTC in wild type cells (Fig. 7A and B). BPA did not promote a significant increase in the shedding of BTC in *Adam17*^{-/-} or *Adam10/17*^{-/-} mEFs (Fig. 7C and D), whereas NP stimulated the release of BTC in *Adam17*^{-/-} (Fig. 7E), but not in *Adam10/17*^{-/-} (Fig. 7F) cells. These findings suggest that ADAM10 is only activated by NP when ADAM17 is absent (i.e. in *Adam17*^{-/-} mEFs), but not when it is present (i.e. in wild type mEFs). Finally, we evaluated whether iRhom1/2 are required for the shedding of BTC induced by NP. Our results showed that BTC release was stimulated by NP (Fig. 7I and J), but not by BPA (Fig. 7G and H) in *iRhom2*^{-/-} mEFs (Fig. 7G and I) or *iRhom1/2*^{-/-} mEFs (Fig. 7H and J). Thus, these findings suggest NP most likely stimulates the release of BTC by activating ADAM10, but only in the absence of ADAM17 (*Adam17*^{-/-} mEFs) or in the absence of iRhom2 or absence of both iRhom1 and iRhom2, which have emerged as key regulators of ADAM17. These results agree with previous results showing that in the absence of ADAM17, ADAM10 is necessary for shedding TGF- α following stimulation

with the ionophore ionomycin (Le Gall et al., 2009). The observation that NP stimulated ADAM10-dependent shedding of BTC, but not ADAM10-dependent shedding of HB-EGF from *Adam17*^{-/-} mEFs, could be explained by the finding that BTC is a highly selective substrate for ADAM10 (Sahin et al., 2004; Sanderson et al., 2005) and thus presumably shed much more efficiently than HB-EGF from *Adam17*^{-/-} mEFs. Moreover, further studies will be necessary to understand why BPA treatment enhanced ADAM10-dependent shedding of TGF α , but not of the ADAM10 substrate BTC in *Adam17*^{-/-} mEFs. Since ADAM10 is known to be regulated by a family of tetraspanin molecules (Matthews et al., 2016), it is conceivable, for example, that distinct ADAM10/tetraspanin complexes are responsible for releasing TGF α versus BTC from *Adam17*^{-/-} mEFs.

Taken together, these results show that BPA and NP can both stimulate the release of EGFR-ligands. These results agree with previous findings showing that BPA is able to induce the release of TNF- α and other EGFR-ligands from different cell types isolated from mice or humans, confirming the physiological relevance of our findings (Ben-Jonathan et al., 2009; Benachour and Aris, 2009; Urriola-Munoz et al., 2014). Since the concentration of xenoestrogens may reach values similar to those used in this study (Li et al., 2010), it is possible that BPA stimulates the release of TNF- α in those cells in a similar way as shown here for HB-EGF, since the shedding of both TNF- α and HB-EGF depends on ADAM17 and iRhom2 (Blobel, 2005; Jackson et al., 2003; Le Gall et al., 2009; Li et al., 2015; McIlwain et al., 2012; Sahin et al., 2004). For instance, mice treated with NP have altered dendritic cell function and altered shedding of TNF- α , suggesting the participation of ADAM17 in this process (Suen et al., 2013). We have shown here that NP stimulates the shedding of HB-EGF and TGF- α even in the absence of ADAM17 or iRhom2, suggesting that NP activates ADAM10. In addition, NP induces the shedding of BTC in mEFs lacking ADAM17 or iRhom1/2, but not in *Adam10/17*^{-/-} mEFs. These results provide new insight on the ability of xenoestrogens to differentially stimulate ADAM17 or ADAM10 depending and the cellular context, and agree with previous observations that stimulated and constitutive ADAM10-dependent shedding of BTC does not require iRhom2 or ADAM17 (Maretzky et al., 2013). Previous studies have also shown that ADAM10 can shed ADAM17 substrates in cells where ADAM17 is inactivated, and under conditions where ADAM10 is activated by other stimuli (Le Gall et al., 2009). In summary, we have identified a new functions of BPA and NP in the potent and differential stimulation of EGFR-ligand shedding. These results provide new insights into the potential mechanism of how the xenoestrogens BPA and NP could affect human health by activation of proinflammatory processes and inappropriate stimulation of cells via activation of iRhoms/ADAM17 or ADAM10.

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Fig. 1. BPA and NP induce TGF-α and HB-EGF shedding in mEF cells

WT mEFs were transfected with the AP-tagged ADAM17 substrates TGF-α (A and B) or HB-EGF (C and D), and then stimulated for 1 h with different concentration of BPA (100, 150, 200, 250 and 300μM) or NP (10, 25, 50, 75 and 100μM) and PMA (25ng/ml) as a positive control. Shedding of TGF-α (A and B) or HB-EGF (C and D) was activated by treatment with BPA 300μM, NP 50, 75 and 100μM and PMA 25ng/ml in WT mEFs.

Vehicle : Ethanol. Results are mean ± SEM; n=6. Statistical analysis were performed before data normalization, mean values were compared using ANOVA and Tukey as a post test; *p 0.05.

Fig. 2. HB-EGF and TGF- α release induced by BPA and NP is dependent on ADAM17
 WT (A–D), *Adam17*^{−/−} (E–H) and *Adam10/17*^{−/−} (I–L) mEFs were transfected with the AP-tagged ADAM17 substrates HB-EGF (A, C, E, G, I and K) or TGF- α (B, D, F, H, J and L) and then activated with BPA (300 μ M), NP (50 μ M) or PMA (25ng/ml), as a positive control, for 1h. BPA (A and B) and NP (C and D) activated ADAM17-mediated shedding, as evidenced by the significantly increase cleavage of HB-EGF (A and C) or TGF- α (B and D) in WT mEFs. Identical experiments were performed with *Adam17*^{−/−} mEFs, in which stimulation for 1 h with BPA (E) or NP (G and H) did not increase the shedding of HB-EGF (E and G) or TGF- α (H) but BPA activated the release of TGF- α (F). However, when *Adam10/17*^{−/−} mEFs transfected with HB-EGF (I and K) or TGF- α (J and L) were stimulated with BPA (I and J) or NP (K and L) did not increase the TGF- α or HB-EGF release. Vehicle : Ethanol. Results are mean $\pm$ SEM; n=3. Statistical analysis were performed before data normalization, mean values were compared using ANOVA and Tukey as a post test; *p $\leq$ 0.05.

Fig. 3. TGF- α release depends on ADAM10 in the absence of ADAM17 induced by BPA
 WT (A) and *Adam17*^{-/-} (B) mEFs were transfected with the AP-tagged ADAM17 TGF- α , and then activated with BPA (300 μ M), NP (50 μ M) or PMA (25ng/ml), as a positive control, for 1h. BPA, NP and PMA-stimulated shedding of TGF- α was strongly inhibited by GW280264X, but not by GI254023X in wild type mEFs (A). Identical experiments were performed with *Adam17*^{-/-} mEFs, in which stimulation for 1 h with NP did not increase the shedding of TGF- α . The BPA-induced release of TGF- α was strongly inhibited by GI254023X, but not by GW280264X (B). Vehicle : Ethanol. Results are mean $\pm$ SEM; n=3. Statistical analysis were performed before data normalization, mean values were compared using ANOVA and Tukey as a post test; *p < 0.05.

Fig. 4. TGF-α and HB-EGF release induced by BPA and NP is independent of iRhom1
 Wild type (A–D) and *iRhom1*^{-/-} (E–H) mEFs were transfected with the AP-tagged ADAM17 substrates TGF-α (A, C, E and G) or HB-EGF (B, D, F and H) and then activated with BPA (300 μM), NP (50 μM) or PMA (25 ng/ml), as a positive control, for 1 h. BPA (A and B) and NP (C and D) activated ADAM17-mediated shedding, as evidenced by the significantly increased cleavage of TGFα (A and C) or HB-EGF (B and D) in wild type mEFs. Identical experiments were performed with *iRhom1*^{-/-} mEFs, in which stimulation for 1 h with BPA (E and F) or NP (G and H) also increase the shedding of TGF-α (E and G) or HB-EGF (F and K). Vehicle : Ethanol. Results are mean ± SEM; n=3. Statistical analysis were performed before data normalization, mean values were compared using ANOVA and Tukey as a post test; *p < 0.05.

Fig. 5. *iRhom2* is required for the stimulated shedding of HB-EGF in response to BPA but not to NP

Wild type (A–D) and *iRhom2*^{-/-} (E–H) mEFs were cotransfected with TGF-α-AP (A, C, E and G) or HB-EGF-AP (B, D, F and H) together with fulllength WT-*iRhom2* or MAD2 as a control, and then stimulated with BPA (300μM), NP (50μM) or PMA (25ng/ml), as a positive control, for 1h. BPA (A and B) and NP (C and D) activated ADAM17-mediated shedding, as evidenced by the significantly increase cleavage of TGF-α (A and C) or HB-EGF (B and D) in wild type mEFs. Identical experiments were performed with *iRhom2*^{-/-} mEFs, in which stimulation for 1 h with BPA (E and F) increased the shedding of TGF-α (E), an *iRhom2*-independent substrate, but not of HB-EGF (F, white bars), an *iRhom2*-dependent substrate, which was recovered when the cells were transfected with the WT-*iRhom2* (F, black bars). However, the stimulation for 1 h with NP increase the shedding of TGF-α (G) and HB-EGF (H). Vehicle : Ethanol. Results are mean ± SEM; n=3. Statistical analysis were performed before data normalization, mean values were compared using ANOVA and Tukey as a post test; *p < 0.05.

Fig. 6. HB-EGF release depends on ADAM10 in the absence of iRhom2 or iRhom1/2 induced by NP

Wild type (A), *iRhom2*^{-/-} (B) and *iRhom1/2*^{-/-} (C) mEFs were transfected with the AP-tagged ADAM17 HB-EGF, and then activated with BPA (300μM), NP (50μM) or PMA (25ng/ml), as a positive control, for 1h. BPA and NP-stimulated shedding of HB-EGF from wild type mEFs was strongly inhibited by GW280264X and GI254023X, but PMA-stimulated shedding of HB-EGF was only inhibited by GW280264X (A). Identical experiments were performed with *iRhom2*^{-/-} (B) and *iRhom1/2*^{-/-} (C) mEFs, in which stimulation for 1 h with BPA did not increase the shedding of HB-EGF, but NP-induced release of HB-EGF was inhibited by GW280264X and GI254023X. Vehicle : Ethanol. Results are mean ± SEM; n=3. Statistical analysis were performed before data normalization, mean values were compared using ANOVA and Tukey as a post test; *p 0.05.

Fig. 7. BTC release is induced by NP only when ADAM17 or iRhom2 or iRhom1/2 are absent
 WT (A and B), *Adam17*^{-/-} (C and E), *Adam10/17*^{-/-} (D and F), *iRhom2*^{-/-} (G and I) and *iRhom1/2*^{-/-} (H and J) mEFs were transfected with the AP-tagged ADAM10 substrate BTC and then stimulated with BPA (300 μM), NP (50 μM) or Ionomycin (IM, 2.5 μM), as a positive control, for 1h. BPA (A, C, D, G and H) did not induce the BTC shedding mediated by ADAM10 in all mEFs cells used. NP (B and F) did not induce the BTC shedding mediated by ADAM10 in WT and *Adam10/17*^{-/-} mEFs, respectively. However, NP induced BTC shedding mediated by ADAM10 in *Adam17*^{-/-} (E), *iRhom2*^{-/-} (I) and *iRhom1/2*^{-/-} (J) mEFs. Vehicle : Ethanol. Results are mean ± SEM; n=3. Statistical analysis were performed before data normalization, mean values were compared using ANOVA and Tukey as a post test; *p < 0.05.