Functional Coupling of Rat Myometrial α1-Adrenergic Receptors to Ghα/Tissue Transglutaminase 2 during Pregnancy*

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Morgan Dupuis, Arlette Lévy, and Sakina Mhaouty-Kodja‡

From the Laboratoire de Physiologie et Physiopathologie, Unité Mixte de Recherche-CNRS 7079, Paris CEDEX 05, France

Ghα protein, which exhibits both transglutaminase and GTPase activities, represents a new class of GTP-binding proteins. In the present study, we characterized Ghα in rat uterine smooth muscle (myometrium) and followed its expression during pregnancy by reverse transcription-PCR and Western blot. We also measured transglutaminase and GTP binding functions and used a smooth muscle cell line to evaluate the role of Ghα in cell proliferation. The results show that pregnancy is associated with an up-regulation of Ghα expression at both the mRNA and protein level. Ghα induced during pregnancy is preferentially localized to the plasma membrane. This was found associated with an increased ability of plasma membrane preparations to catalyze Ca2+-dependent incorporation of [3H]putrescine into casein in vitro. In the cytosol, significant changes in the level of immunodetected Ghα and transglutaminase activity were seen only at term. Activation of α1-adrenergic receptors (α1-AR) enhanced photofinity labeling of plasma membrane Ghα. Moreover, the level of α1-AR-coupled Ghα increased progressively with pregnancy, which parallels the active period of myometrial cell proliferation. Overexpression of wild type Ghα in smooth muscle cell line DDT1-MF2 increased α1-AR-induced [3H]thymidine incorporation. A similar response was obtained in cells expressing the transglutaminase inactive mutant (C277S) of Ghα. Together, these findings underscore the role of Ghα as a signal transducer of α1-AR-induced smooth muscle cell proliferation. In this context, pregnant rat myometrium provides an interesting physiological model to study the mechanisms underlying the regulation of the GTPase function of Ghα.

Different from the traditional heterotrimeric and monomeric G proteins, Ghα protein is a bifunctional enzyme with both transglutaminase and GTPase activities (1). Transglutamination requires Ca2+ and is inhibited by GTP, whereas GTPase activity is inhibited by Ca2+. Initially identified as tissue transglutaminase 2 (TG2),1 Ghα is a member of a large family of transglutaminases that includes plasma Factor XIIIa, epidermal TG1 and TG3, or prostate TG4 (2). These enzymes catalyze the formation of covalent γ-glutamyl-ε-lysine bonds between proteins or polyamines and protein substrates. Such modifications play a role in different biological processes such as blood coagulation, epidermal differentiation, formation of copulatory plug in rodents, or extracellular matrix organization (2). Like other transglutaminases, Ghα consists of four domains: an amino-terminal β-sandwich and a catalytic core with the active site cysteine (Cys-277) for transglutamination, followed by two carboxyl-terminal β-barrels. The three-dimensional structure of Ghα complexed with GDP revealed a unique guanine nucleotide binding pocket located between the catalytic core and the first β-barrel and formed by residues coming from both domains (3). By virtue of its GTP binding/GTPase activity, Ghα acts as a signaling molecule for α1-AR (4, 5), oxytocin receptors (6, 7), and thromboxane receptors (8). α1-AR were, however, the most studied Ghα protein-coupled receptors. Activation by α1-AR induces exchange of GDP to GTP and dissociation of GTP-Ghα from the Ghβγ subunit, which was recently identified as calreticulin (9). GTP-bound Ghα interacts with downstream effector PLCβ1, thereby resulting in phosphoinositide hydrolysis and Ca2+ increase (10–13). Deactivation of this signaling pathway is triggered by GTP hydrolysis and reassociation of Ghα with free Ghβγ subunit. Although α1-AR/Ghα coupling was well established in many cell types, the cellular responses triggered by this signaling pathway still remain unclear. Indeed, only a few studies have addressed the role of Ghα as a signal transducer of G protein-coupled receptors.

The present study was undertaken to investigate the potential involvement of Ghα in myometrial α1-adrenergic-induced responses. It is well known that norepinephrine modulates myometrial contractility during pregnancy. Adrenergic signaling pathways are under the control of progesterone and estradiol, which regulate the expression of receptors (β-AR), heterotrimeric G proteins (Gαi, Gαq, and Gqα), and PLCβ enzymes (14–17). Hence, during pregnancy under progesterone dominance, norepinephrine stimulates β-AR and α2-AR, which both activate adenylyl cyclase and increase intracellular concentrations of cAMP (18, 19). The latter second messenger induces myometrial relaxation by inhibiting the pathways leading to Ca2+ increase (20). At term, when concentrations of estradiol rise, the β-adrenergic pathway becomes desensitized (21), and norepinephrine acts on α2-AR (19, 22) and α1-AR (23). At this time, α2-AR shift to inhibitors of adenylyl cyclase, whereas α1-AR activate the GqPLCβ system and participate in the Ca2+ increase and uterine contraction. An intriguing observation is that α1-AR are expressed throughout pregnancy, although they are not efficiently coupled to phosphoinositide hydrolysis due to down-regulation of Gqα (16) and PLCβ isoforms at this period (17). We thus questioned whether these receptors could signal through Ghα to regulate response(s) other than contraction. Indeed, myometrial cells undergo proliferation during pregnancy, which is critical for uterine adap-

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‡ To whom correspondence should be addressed. Present address: Institut Pasteur, Département de Biologie du Développement, Bâtiment Jacques Monod, 25, rue du Docteur Roux, 75724 Paris CEDEX 15, France. Tel.: 331-45-68-84-96; Fax: 331-40-61-31-09; E-mail: smhaouty@pasteur.fr.

The abbreviations used are: TG, transglutaminase; AR, adrenergic receptors; PLC, phospholipase C; PVDF, polyvinylidene difluoride; AppNH2, adenylyl-5-yi imidodiphosphate; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
tation to fetoplatental growth (24, 25). Although many studies have addressed the regulation of such a process in physiopathological situations like uterine leiomyoma, very little is known about the physiological processes involved during pregnancy.

For this purpose, we first characterized myometrial Ghα and followed its expression, at the mRNA and protein level, during pregnancy and at term. We also determined myometrial transglutaminase activity and photoaffinity labeling of Ghα in the absence or presence of GTP or α1-adrenergic agonist. Finally, we used a smooth muscle cell line to analyze the role of Ghα and its transglutaminase-inactive (C277S) mutant in cell proliferation induced by α1-AR.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Thymidine (7 Ci/mmol), [α-32P]GTP (3000 Ci/mmol), and polyvinylidene difluoride (PVDF) membranes were purchased from PerkinElmer Life Sciences. [3H]Putrescine (33 Ci/mmol) and enhanced chemiluminescence reagent were from Amersham Biosciences. Applied Biosystems, and primer sets were used as control. DDT1-MF2 cells were then given fresh serum-free medium and allowed to achieve quiescence for 24 h prior to subsequent treatments. DNA synthesis was assessed by Student’s t test for unpaired data. A probability level less than 0.05 was considered statistically significant.

**RESULTS**

**Characterization of Rat Myometrial Ghα**—To assess the expression of Ghα in pregnant rat myometrium, we used RT-PCR technique and Western blot analysis. Rat liver where the expression of Ghα has been reported already was used as control. Using specific primers, we amplified a 520-bp fragment of Ghα from pregnant rat myometrium and rat liver (Fig. 1A, lanes 2 and 4). Control PCR reactions performed on nontranscribed RNAs indicated no contamination of the RNA preparations with genomic DNA (Fig. 1A, lanes 1 and 3). Monoclonal antibodies raised against Ghα stained a single band of the appropriate molecular mass (74 kDa) in plasma membranes and cytosolic fractions of pregnant rat myometrium and rat liver (Fig. 1B). Our results also show that the subcellular distribution of Ghα is tissue-specific (Fig. 1B). Indeed, Ghα is predominantly cytosolic in liver, while a high amount of this protein is associated with the myometrial plasma membranes.
expression level of myometrial Gha at different stages of pregnancy. Semiquantitative analysis showed that the level of Gha transcripts, but not of the internal control GAPDH, is altered during pregnancy (Fig. 2A). Indeed, mRNA level of Gha increased progressively during pregnancy, with pronounced changes occurring at day 12 of pregnancy (2-fold of day 5) and at term (1.5-fold of day 20) (Fig. 2B). Quantification of the immunodetected Gha also indicated an up-regulation of this protein. Indeed, the amount of membrane-associated Gha increased progressively during pregnancy (Fig. 3A). In contrast, the amount of cytosolic Gha remained stable until day 20 of pregnancy (Fig. 3B), and a significant elevation (+35%) was seen only at term. Comparison of the subcellular distribution of Gha during pregnancy was expressed as the membrane/cytosolic ratio. The obtained data illustrated in Fig. 3C revealed a progressive enrichment of plasma membrane compartment in Gha during the course of pregnancy. Indeed, whereas the cytosolic localization of Gha prevailed over membrane-associated Gha at day 5 of pregnancy, this situation was completely reversed at the end of pregnancy (ratio of 0.56 at day 5 versus 1.5–1.8 between day 20 and term).

Altogether, these results indicate an up-regulation of rat myometrial Gha during pregnancy. Moreover, this period is associated with a preferential increase of membrane-bound Gha.

Myometrial Transglutaminase Activity during Pregnancy—
Gha is a bifunctional enzyme with both transglutaminase and GTPase activities. To characterize myometrial transglutaminase activity, we tested the ability of plasma membrane and cytosolic fractions to catalyze, in vitro, the incorporation of [3H]putrescine into dimethylcasein. Addition of 2 mM CaCl2 increased transglutaminase activity in both plasma membrane and cytosolic fractions of pregnant rat myometrium (6.4-fold and 4.8-fold above basal in the plasma membrane and the cytosol, respectively) (Fig. 4A). To evaluate the degree of participation of Gha in this myometrial activity, we tested the inhibitory effect of GTP. As shown in Fig. 4A, 0.5 mM GTP blocked ~90% of Ca2+-stimulated transglutaminase activity in plasma membrane and cytosolic compartments.

We also examined myometrial transglutaminase activity at different stages of pregnancy. In the absence of Ca2+, plasma membrane and cytosolic transglutaminase activity did not change throughout pregnancy (a mean of 121 and 66 fmol/mg of protein/min in plasma membrane and cytosol, respectively). Addition of Ca2+ significantly increased transglutaminase activity, and GTP counteracted this elevation in both fractions at all stages of pregnancy studied (data not shown). As illustrated in Fig. 4, B and C, Ca2+-stimulated activity of the plasma membrane fraction increased progressively during pregnancy, whereas the cytosolic transglutaminase activity was stable until day 20 of pregnancy. In correlation with Western blot analysis, a significant increase (+30%) of the cytosolic transglutaminase activity was observed only at term (Fig. 4C).

Functional Coupling of Myometrial α1-AR to Gha—Next we analyzed whether myometrial Gha, by virtue of its GTP binding activity, interacts with α1-AR. For this purpose, plasma membrane preparations were incubated with [α-32P]GTP in the absence or presence of 10 μM phenylephrine (α1-adrenergic agonist). Labeled G proteins were then separated by electrophoresis and transferred to PVDF membranes prior to autoradiography and immunodetection. The molecular mass of the 74-kDa radiolabeled protein coincided with that of the immunoreactive Gha detected by Western blot analysis (Fig. 5A, top and bottom). Addition of unlabeled GTP completely blocked labeling of Gha (Fig. 5A, top). Moreover, application of phenylephrine significantly augmented photoaffinity labeling of Gha (140–160% of basal) at all stages of pregnancy studied (Fig. 5, A (top) and B). These responses were blocked by addition of phentolamine, an α1-adrenergic antagonist (data not shown). Interestingly, both basal and phenylephrine-stimulated [α-32P]GTP labeling of Gha increased during pregnancy (Fig. 5B). The maximal amounts were observed between day 20 of pregnancy and term. In this comparative study, labeled Gha was hardly detectable at day 5 of pregnancy, even in the presence of phenylephrine.

Gha in DDT1-MF2 Smooth Muscle Cell Line—α1-AR/Gha coupling was shown to activate PLCδ1, thereby resulting in phosphoinositide hydrolysis and Ca2+ increase (11, 13). To investigate whether such activation occurs in pregnant rat myometrium, we first characterized the expression of PLCδ1. Results illustrated in Fig. 6A indicated the presence of this PLC enzyme in non-pregnant, pregnant, and term rat myometrium. However, the amount of PLCδ1 during pregnancy represents only 30% of that observed in term rat animals (Fig. 6B). Pregnancy-dependent down-regulation of PLCδ1 is in correlation with the fact that all pathways leading to Ca2+ increase and contraction are inhibited or reduced during pregnancy. We thus asked whether α1-AR/Gha coupling could be involved in myometrial proliferation. To answer this question, we used the DDT1-MF2 cell line, which was described previously as a useful model to study the myometrial adrenergic signaling pathway regulations (31, 32). As myometrial cells, this smooth
FIG. 3. **Myometrial Gα expression during pregnancy.** A and B, quantification of myometrial plasma membrane (PM) and cytosolic (CYT) Gα at days 5 (d5), 12 (d12), 15 (d15), and 20 (d20) of pregnancy and at term (T). Data are expressed as percentage of T and are mean ± S.E. of nine independent experiments. The amount of plasma membrane and cytosolic Gα in non-pregnant rat represented 50 ± 7% and 60 ± 6% of term value, respectively. a, p < 0.05 compared with expression at d5; b, p < 0.05 compared with expression at d12 or d15. C, ratio of membrane/cytosolic Gα during pregnancy (d5, d12, d15, and d20) and at T. Data are mean ± S.E. of four independent experiments. a, p < 0.05 compared with d5; b, p < 0.05 compared with d12. c, p < 0.05 compared with d15.

![Image](https://example.com/image1.png)

**FIG. 4.** **Myometrial transglutaminase activity during pregnancy.** A, plasma membrane (PM) and cytosolic (CYT) fractions of rat myometrium at day 12 of pregnancy were assayed for transglutaminase activity in the absence (Basal) or presence of CaCl2 or CaCl2 plus GTP. Data are mean ± S.E. of three independent experiments. a, p < 0.001 compared with basal, b, p < 0.05 compared with CaCl2, B and C, Ca2+ stimulated transglutaminase activity in myometrial plasma membrane (B) and cytosolic (C) fractions during pregnancy (d5, d12, d15, and d20) and at term (T). Data are expressed as percentage of T and are mean ± S.E. of nine independent experiments. Plasma membrane and cytosolic transglutaminase activities in non-pregnant rat represented 45 ± 0.3% and 74 ± 2% of term value, respectively. a, p < 0.05 compared with d5; b, p < 0.05 compared with d12.

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**DISCUSSION**

**Expression of Gα in Rat Myometrium**—By using RT-PCR and Western blot analysis, we showed the expression of Gα in pregnant and term rat myometrium. Transglutaminase assay

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also indicated the presence of a Ca2+- and GTP-sensitive transglutaminase. In addition, GTP inhibited ~90% of myometrial transglutaminase activity. Among members of the transglutaminase family, TG3 is also sensitive to guanine nucleotides (34). However, this transglutaminase presents a restricted expression to epidermis (2). Based on these observations, we can then easily conclude that Gα is predominantly expressed in pregnant and term rat myometrium. Another argument in favor of the predominant expression of Gα in pregnant rat myometrium is the good correlation among the levels of immunodetected Gα protein, myometrial transglutaminase activi-
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Fig. 5. Photoaffinity labeling of myometrial \( \text{G} \alpha \) during pregnancy. A, top, autoradiography of \( [\alpha-^{32}\text{P}]\)GTP-photolabeled myometrial membrane preparations from rat at day 20 of pregnancy in the absence (Basal) or presence of phenylephrine (Phe) or unlabeled GTP. Bottom, the presence of \( \text{G} \alpha \) was analyzed in the same gel with the monoclonal anti-\( \text{G} \alpha \). B, quantification of \( [\alpha-^{32}\text{P}]\)GTP-photolabeled \( \text{G} \alpha \) during pregnancy (d12, d15, and d20) and at term (T) in the absence (Basal) or presence of phenylephrine (Phe). Results are expressed as percentage of basal term values and are mean ± S.E. of three independent experiments. * \( p < 0.05 \) compared with basal at d12 or d15. ** \( p < 0.05 \) compared with its corresponding basal.

Fig. 6. Characterization and quantification of myometrial PLC\( \delta 1 \). A, representative Western blot of cytosolic PLC\( \delta 1 \) in non-pregnant (NP), pregnant at day 12 (P), and term (T) rat myometrium. B, the average results expressed as percentage of term values. Data are mean ± S.E. of four independent experiments. The amount of PLC\( \delta 1 \) at days 5, 15, and 20 of pregnancy was not statistically different from that described at day 12. * \( p < 0.0001 \) compared with T.

Fig. 7. Overexpression of \( \text{G} \alpha \) increased proliferation of DDT1-MF2 cells. Cells were transiently transfected with constructs of wild type (WT) and transglutaminase-inactive (C277S) mutant of \( \text{G} \alpha \) or empty vector (Control). A, Western blot to detect the expression of \( \text{G} \alpha \). B, transglutaminase activity in transfected DDT1-MF2 cells in the presence of 2 mm CaCl\(_2\). C, cells were incubated for 24 h in the absence (Basal) or presence of phenylephrine (Phe). Cell proliferation was determined after \([^{3}H]\)thymidine incorporation as described under “Experimental Procedures.” Data are expressed as percentage of basal control cells and are mean ± S.E. of three independent experiments performed in triplicate. * \( p < 0.05 \) compared with its corresponding basal. ** \( p < 0.05 \) compared with Phe in control cells.

The presence of \( \text{G} \alpha \) was also reported in non-pregnant human myometrium (6). However, its regulation has not been studied throughout pregnancy. The present study shows that the mRNA and protein levels of \( \text{G} \alpha \) increase with pregnancy. This suggests that myometrial \( \text{G} \alpha \) gene is regulated, at least in part, at the transcriptional level. Our preliminary experiments indicate that myometrial \( \text{G} \alpha \) is not under the control of progesterone or estradiol (data not shown). A possible candidate for such a regulation could be retinoic acid. Indeed, this potent inducer of \( \text{G} \alpha \) expression, as shown in many cell types, is locally synthesized and stored in pregnant rat uterus (36, 37). In addition, rat myometrium expresses different retinoic acid receptors (RAR\( \alpha \), RAR\( \beta \), and RXR\( \beta \)) that could transduce retinoic acid-induced effects (37, 38). Future studies will examine whether myometrial \( \text{G} \alpha \) is regulated by retinoic acid during pregnancy.

Preferential Association of Induced \( \text{G} \alpha \) with Myometrial Plasma Membranes during Pregnancy—\( \text{G} \alpha \) is known to reside predominantly in the cytosol. However, some studies localized a portion of this protein in the plasma membrane compartment (2). The present study reports a progressive enrichment of myometrial plasma membranes in \( \text{G} \alpha \) during pregnancy. Indeed, Western blot analysis and transglutaminase assay as well as photoaffinity labeling study showed an increase of plasma membrane-associated \( \text{G} \alpha \), whereas the amount of \( \text{G} \alpha \) in the cytosol remained unchanged until day 20 of pregnancy.
The molecular mechanisms underlying this differential localization of intracellular Gha during pregnancy need to be clarified. It is possible that Gha actively translocates from the cytosol to the plasma membrane during pregnancy because this protein was reported to moonlight between these compartments (39, 40). Alternatively, retinoic acid was shown to increase the ability of Gha to associate with the plasma membrane in HeLa cells (41).

Interaction between Myometrial a1-AR and Gha—By using photoaffinity labeling technique, we showed that myometrial Gha functionally interacts with a1-AR in pregnant and term rats. The progressive enhancement in the level of a1-AR-coupled Gha correlates well with the up-regulation of plasma membrane-associated Gha during pregnancy. This strongly suggests that plasma membrane-associated Gha is accessible to the interaction with G protein-coupled receptors in pregnant rat myometrium. Previous findings in human vascular smooth muscle cells reported that the particulate Gha codistributes with stress fibers and may thus stabilize cytoskeletal structures through its cross-linking function (42). In the latter study, the particulate Gha appeared inactive when assayed by in vitro putrescine/casein assay, maybe due to its tight binding to preferred local substrates (42). It is known that stabilization of transglutaminase activity into cells requires both high concentrations of Ca2+ and a decrease of guanine nucleotide levels (43, 44). In rat myometrium, pathways leading to Ca2+ increase and uterine contraction are inhibited during pregnancy to allow development of fetoplacental units. In addition, in the presence of exogenous Ca2+, myometrial plasma membrane Gha was able to catalyze the incorporation of putrescine into casein. These discrepancies between both models lead us to suggest that plasma membrane Gha plays different roles in uterine and vascular smooth muscles.

The present work supports previous findings in that intracellular localization of Gha dictates its functions. Indeed, Gha purified from plasma membranes was shown to exhibit higher GTP binding activity than the cytosolic Gha in mouse heart (45). In addition, translocation of Gha from the plasma membrane to the cytosol is accompanied by the loss of GTP binding and the appearance of transglutaminase activity (39, 40). In myometrium, the up-regulation of cytosolic Gha at term coincides with the onset of labor. At this time, the very high elevation of intracellular concentrations of Ca2+ could stimulate transglutaminase activity. Recent data from mice lacking TG2 showed that this function is important for the stabilization of apoptotic thymocytes before their clearance (46). Therefore, it is tempting to suggest that the cytosolic Gha stabilizes dying myometrial cells during uterine involution that occurs after delivery.

Gha-induced Smooth Muscle Cell Proliferation—We have shown previously that myometrial a1-AR participate in the initiation of uterine contraction at term through activation of the Gq/PLC system (23, 47). During pregnancy, Gqa and PLCβ isoforms are down-regulated, thereby resulting in a weak phosphoinositide hydrolysis in response to the activation of a1-AR (16, 17). The present study shows that a1-AR interact with Gha in pregnant rat myometrium. Moreover, the level of a1-AR-coupled Gha increased during pregnancy, with a peak reached at day 20. Interestingly, our recent findings reveal a similar pattern for the increase of the myometrial weight and DNA amount during pregnancy. The correlation between a1-AR/Gha coupling and myometrial proliferation could be highly relevant to understanding the physiological significance of a1-AR expression during pregnancy, particularly given that a1-AR mediate proliferation of several smooth muscle cell types (48).

To address this question, we transfected DDT1-MF2 cells with wild type and transglutaminase-inactive Gha. Previous studies have shown that mutating the active site cysteine (Cys-277) impairs transglutaminase activity of Gha without affecting its GTP binding/GTPase function and interaction with a1-AR (49). The results indicated that a1-AR operate through Gha to stimulate smooth muscle cell proliferation. Firstly, overexpression of Gha enhanced a1-adrenergic-induced DNA synthesis. Secondly, the use of C277S-Gha showed that this response involves the GTP binding/GTPase function of Gha. Some studies in vascular smooth muscle cells reported the involvement of heterotrimeric G proteins in a1-AR-induced proliferation (48). In the present work, we describe an additional mechanism by which a1-AR could regulate smooth muscle cell proliferation. Indeed, our data are the first demonstration of the involvement of Gha in such a cellular response. The molecular mechanisms underlying smooth muscle cell proliferation remain to be clarified. In rat hepatocytes, it was suggested that Gha could act on PLCβ1 to induce cell proliferation (50). In non-pregnant human myometrium, such interaction between Gha and PLCβ1 was described (7). Nevertheless, our results indicate that if PLCβ1 could be a downstream effector for a1-AR/Gha coupling and participate in the Ca2+ increase and uterine contractions at term, this seems unlikely during pregnancy. In fact, at this period, PLCβ1 is down-regulated, as are the majority of the contraction-associated proteins. Gha was also shown to participate in the activation of extracellular signal-regulated kinases by a1-AR in neonatal rat cardiomyocytes (51). Further studies will define whether Gha activates the mitogen-activated protein kinase pathway to induce myometrial cell proliferation during pregnancy.

In summary, our results reveal, for the first time, that the expression of Gha is induced in pregnant rat myometrium. Moreover, the induced protein preferentially associates with the plasma membrane where it interacts with a1-AR. During pregnancy, Gha may play an important role in the transduction of myometrial a1-adrenergic signaling. Firstly, we have shown previously that Gqα is down-regulated at this period. Secondly, Gha enhanced a1-adrenergic-induced proliferation of DDT1-MF2 smooth muscle cell line.

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Morgan Dupuis, Arlette Lévy and Sakina Mhaouty-Kodja

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