Basic FGF decreases clearance receptor of natriuretic peptides in fetoplacental artery endothelium

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Itoh, Hiroaki, Jing Zheng, Ian M. Bird, Kazuwa Nakao, and Ronald R. Magness. Basic FGF decreases clearance receptor of natriuretic peptides in fetoplacental artery endothelium. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R541–R547, 1999.—Atrial natriuretic peptide (ANP) is present in the fetoplacental circulation of humans and sheep. The ANP-A receptor is the specific membrane receptor for ANP, which produces cGMP. The clearance receptor of natriuretic peptide (CR) is postulated to modulate local concentrations of ANP, thereby modulating cGMP production through the ANP-A receptor. Recently we reported that fetoplacental basic fibroblast growth factor (bFGF) and cGMP levels are increased dramatically during the third trimester of ovine gestation. Therefore we hypothesized that bFGF will downregulate CR expression in cultured ovine fetoplacental artery endothelial (OFPAE) cells via the mitogen-activated protein kinase (MAPK) signal cascade mechanism, thereby causing augmentation of ANP-mediated cGMP production. Western analysis and/or RT-PCR of CR expression were performed after treatment of OFPAE cells with bFGF (10 pg/ml–1 µg/ml) with or without 50 µM PD-98059, a selective inhibitor of MAPK kinase. To investigate possible effects of CR downregulation on the functional modulation of ANP-A receptor activation, cGMP production (20 min) by OFPAE cells was measured in response to ANP (10 pM–1 µM) with or without pretreatment (24 h) of 10 ng/ml bFGF. CR expression in OFPAE cells was dose dependently downregulated by 1–10 ng/ml bFGF treatment (maximum – 69%), which was completely reversed by pretreatment with PD-98059. Treatment of OFPAE cells with 10 ng/ml bFGF (24 h) did not alter maximum ANP-A activity (cGMP production/20 min), but decreased the apparent ED50 of ANP to stimulate cGMP production from 2.5 to 0.83 nM, suggesting the possibility that bFGF-mediated downregulation of CR may elevate ANP-mediated cGMP production responses. Thus bFGF downregulates CR mRNA and protein expressions via the MAPK cascade in OFPAE cells.

ATRIAL NATRIURETIC PEPTIDES (ANP) are present in the fetoplacental circulation in humans (14) and sheep (27), and ANP concentrations are elevated in fetal distress (12, 14). The biologically active receptors of natriuretic peptides have a guanylyl cyclase (GC) domain that constitutes a major part of the particulate guanylyl cyclases of the arterial wall. This GC domain synthesizes cGMP as an intracellular second messenger, which causes potent vasorelaxation (9, 21, 24, 25). There are two biologically active receptors of natriuretic peptide, the ANP-A receptor [particulate guanylyl cyclase-A (pGC-A)] and the ANP-B receptor (pGC-B). The ANP-A receptor (pGC-A) is the specific receptor for ANP and the ANP-B receptor (pGC-B) is the specific receptor for C-type natriuretic peptide (CNP; 25, 30). ANP-A (pGC-A) and ANP-B (pGC-B) receptors are present in uterine tissues from pregnant women (13).

The clearance receptor of natriuretic peptide (CR) has little or no guanylyl cyclase domain (24, 30). Moreover, CR is postulated to regulate the local concentrations of natriuretic peptides thereby modulating ANP-A/B (pGC-A/B) activities (3, 5, 15, 16, 19, 24, 30). Pregnancy downregulates CR protein expression in ovine uterus, but not systemic arteries (11). Because CR has only a short intracellular GC domain, whereas pGC-A/B have extensive intracellular GC domains, there is controversy over whether CR may also function as a signaling receptor. Cohen et al. (5) and Maack (19) demonstrated that CR does not function as a signaling receptor to modify the major known cardiovascular, adrenal, and renal effects of natriuretic peptides. In contrast, Prins et al. (26) reported that CR alters the mitogen-activated protein kinase (MAPK) signal cascade or production of cAMP (10), although it is still unclear how CR couples with these signaling pathways, without an extensive intracellular GC domain.

Ovine fetoplacental basic fibroblast growth factor (bFGF) production increases dramatically during the third trimester (34) concomitant with parallel increases of amniotic fluid cGMP levels (29). Subsequently we have demonstrated that bFGF increased the expression of endothelial nitric oxide synthase (eNOS) via the MAPK signal cascade in ovine fetoplacental artery endothelial OFPAE cells (33). Because nitric oxide (NO) exerts its biological activity in vascular smooth muscle cells via activation of soluble guanylate cyclase, which produces cGMP (6), our previous observations suggest the interesting possibility that bFGF contributes, at least partly, to the augmentation of cGMP production by ovine placental vasculature. Previously we reported that ANP-A/B (pGC-A/B) as well as CR are not only present in vascular smooth muscle cells but also endothelial cells (32). Therefore, in addition to the previously reported (29, 33) upregulation of eNOS expression, in the current study we hypothesized that bFGF increases the production of cGMP in response to pGC-A/B stimulation by modulating pGC-A/B maximum activities or CR expression in...
OFPAE cells, thereby contributing to the increase in cGMP production in ovine placenta. The specific objectives of the current study were to evaluate in OFPAE cells: 1) if bFGF downregulates expression levels of CR protein as well as mRNA; 2) if pretreatment with PD-98059, a selective inhibitor of MAPK kinase (Calbiochem, San Diego, CA; 1, 7), inhibits the downregulation of CR by bFGF; 3) if bFGF has any effect on the maximum activities of ANP-A receptors (pGC-A); and 4) if bFGF treatment enhances the ability of ANP to stimulate cGMP production via the downregulation of CR.

MATERIALS AND METHODS

All reagents used were of analytic grade and were purchased from Sigma (St. Louis, MO), unless otherwise stated. Experimental protocol. OFPAE cells were isolated from ovine fetoplacental artery and validated recently in our laboratory (33, 35). The OFPAE cells were maintained in DMEM containing 10% calf serum and 10% fetal bovine serum. Cells were seeded on 12-well plates for guanylate cyclase (GC) activity assays and 60 × 15 mm culture dishes for CR quantification by Western blot analysis and total RNA extraction. The cultures were conducted under 5% CO2 in air at 37°C. When the cells reached 90% confluence, the media was changed with fresh DMEM containing no serum and incubated for 24 h. Then, the media was changed with fresh DMEM in the absence or presence of various concentrations (100 fg/ml–1 µg/ml) of bFGF (R&D Systems, Minneapolis, MN) for 8–36 h before the GC activity assay. Western immunoblot, or total RNA extraction. Before the GC activity assay, wells were washed with 10 mM PBS. Pretreatment with PD-98059 (50 µM), a selective inhibitor of MAPK, started 1 h before adding bFGF, and Western immunoblot analysis of CR was determined after 24 h of bFGF treatment.

Western immunoblot analysis of the CR. Western immunoblot analysis of ovine CR protein expression was performed as we previously described (11). OFPAE cells in culture dishes were scraped in 100 µl solubilizing buffer (150 mM NaCl, 50 mM Tris·HCl, 10 mM EDTA (pH 7.4), 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptine, 5 µg/ml aprotinin) and were sonicated and then centrifuged (12,250 g) for 10 min. The supernatant was fractionated by 7.5% SDS polyacrylamide gel electrophoresis (10 µg/lane, 100 V, 2 h) with Rainbow molecular weight markers (Bio-Rad Laboratories) before transfer to Immobilon P membrane (100 V, 2 h). Immunodetection was achieved by using a mouse monoclonal antibody, raised against bovine CR (11, 16) (1:1,000; 1 h, room temperature) and followed by enhanced chemiluminescence reagent system with exposure to hyperfilm (Amersham, Arlington Heights, IL).

RT-PCR analysis of ovine CR mRNA mass assay. The RT-PCR analysis of ovine CR mRNA expression was performed as we described previously (11). Ovine CR cDNA partial clones were kindly donated from Peter Aldred, University of Melbourne. Total RNA was extracted from OFPAE cells, using a phenol-chloroform-isoamyl alcohol extraction procedure as reported previously (2, 4, 33). CR mRNA were quantified by coupled RT-PCR amplification in a single tube, as recently described (2, 4). For mRNA quantification, total RNA (0.5 µg/tube) was incubated in a 50 µl final volume containing 1× PCR buffer [2 mM MgCl2, 10 nmol of each dATP, dCTP, dTTP, and dGTP ( Gibco BRL, Grand Island, NY); 30 pmol forward and reverse temperature-matched primers; 1 µl (2.5 U) AMV reverse transcriptase (Gibco BRL); and 1 µl (5 U) of Taq polymerase transcriptase (Gibco BRL)]; RT controls only contained Taq polymerase. The forward and reverse primers, used for targeting amplification from part of the ovine CR protein coding region (8), were: 5’-TACTGTGAACTCAGACGTG-3’/5’-AGTAACTCACAATCTCCTG-3’, respectively. The expected final products from ovine CR mRNA were 192 bases. The program used was anneal 62°C, 10 min; reverse transcription 50°C, 10 min; denature 94°C, 2 min; amplify 28 cycles using 94°C, 30 s; 55°C, 30 s; 72°C, 30 s. Final products were extended to full length by incubation at 72°C for 30 s. Controls for each assay included total RNA extracted from ovine kidney and a standard curve containing known copy numbers of CR cDNA target sequences, respectively. At the end of the assay, 10 µl of products were separated on a 2% agarose TAE gel and transferred to MagnaGraph hybridization membrane (Molecular Separations, Westborough, MA) for Southern blotting (against probes generated from pOCHR using asymmetric PCR; 2, 4). After hybridization, membranes were washed once in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)-0.1% SDS for 15 min and twice in 0.1× SSC-0.1% SDS (2× 30 min) before drying and direct exposure to a phosphorimage (Bio screen, Bio-Rad Laboratories, Hercules, CA; 5 min) for direct quantification (Molecular Analysis v1.4, Bio-Rad Laboratories). Data were normalized to ovine GAPDH RNA expression also measured by RT-PCR and expressed as copy number per milligram RNA.

GC activity assay. The activities of soluble GC (sGC) and particulate GC-A/B (pGC-A/B) were estimated as the production of cGMP from OFPAE cells, as we described previously (11, 20). Each experiment was performed in triplicate wells. After washing with 10 mM PBS, OFPAE cells in 12-well plates were incubated (20 min) under atmosphere of 5% CO2 in air at 37°C for 20 min in DMEM either alone (control) or with the maximal stimulating dose of 100 µM sodium nitroprusside (SNP); the NO donor used as an sGC stimulant), 10 µM 1-µM human ANP (1–28) (the specific ligand of pGC-A), or the maximal stimulating dose of 1 µM human CNP (1–22) (Peptide Institute, Minoh, Japan). The incubation time and maximal stimulating doses of each treatment group were based on our previous reports (11, 13, 20, 30–32). The incubation was performed using 0.5 mL/well serum-free DMEM containing 100 µM isobutylmethylxanthine for phosphodiesterase inhibition and 0.01% BSA (crystallized) for natriuretic peptide delivery to cells. After 20 min of incubation, the reaction was terminated by adding 0.1 mL of ice-cold 36% TCA, and the mixture of media and cell protein was collected and stored at −20°C. TCA and protein were separated from the mixture by mixing with 120% (vol/vol) of a mixture (1:1) of trioctylamine (Aldrich, Milwaukee, WI):1,1,2-trichlorotrifluoroethane, and cGMP content of the aqueous phase was determined by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) (11, 20). The total cGMP content in the mixture divided by total cell number per well was regarded as sGC and/or pGC activities.

Statistical analysis. The Mann-Whitney U test was used for the RT-PCR data. Other statistical analyses performed were ANOVA followed by Fisher’s protected least significant difference test. Values are presented as means ± SE. P < 0.05 was regarded as significant.

RESULTS

Effects of bFGF on expression of CR protein in OFPAE cells. Western immunoblot analysis of CR revealed a 65-kDa main band and a 67-kDa minor band (see Figs. 1 and 3). Recently we reported that Western immunoblot analysis showed only 65-kDa CR bands in preg-
nant and nonpregnant ovine uterine, omental, and renal arteries as well as kidney (11). At present we do not know whether the 67-kDa minor band represents CR specific for fetal tissue or OFPAE cells. The percentage changes of the protein expression of CR (calculated as % of control in arbitrary units from 4 different blots) after treatment (24 h) with 10 pg/ml, 100 pg/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1 µg/ml bFGF were 112 ± 37, 113 ± 41, 59 ± 32 (P < 0.05 vs. control), 31 ± 11% (P < 0.05), 48 ± 11 (P < 0.05), and 82 ± 29% (Fig. 1B). Therefore a maximum 69% decrease of CR protein expression was observed with 10 ng/ml bFGF treatment. The downregulation of CR protein expression by 10 ng/ml bFGF was not observed until after 8 h of treatment (Fig. 1C).

Effects of bFGF on mRNA expression of CR in OFPAE cells. Figure 2A shows the standard curve of RT-PCR, using known numbers of copies of CR cDNA templates. We observed an excellent correlation (r = 0.987, P < 0.0001) between the log(counts arbitrary units) and log(copy numbers of CR/tube). Southern blot analysis of the CR RT-PCR products derived from total RNA obtained from OFPAE cells and ovine kidney (positive control) showed the expected-sized bands of 192 base pairs (Fig. 2B). Therefore the expected sensitivity of the RT-PCR assay was 4.1 × 10^3 copies of CR mRNA per microgram total RNA. No signals were observed in RT(−) kidney total RNA (Fig. 2B). Treatment with bFGF dose dependently decreased CR mRNA expression. After treatment (24 h) with 10 and 100 ng/ml bFGF, the copy number of CR mRNA/µg total RNA was significantly lower than that of control (25 and 63% vs. control, respectively; P < 0.05; Fig. 2C). In contrast to the maximal mRNA response observed at 100 ng/ml bFGF, Western immunoblot analysis showed that maximum downregulation of CR protein expression was observed with 10 ng/ml bFGF treatment (Fig. 1).

Effects of PD-98059 on bFGF-induced CR downregulation in OFPAE cells. Pretreatment with 50 µM PD-
98059 alone had no effect on CR protein levels, whereas PD-98059 completely inhibited the bFGF-induced down-regulation of CR protein levels in OFPAE cells, suggesting that MAPK cascade is involved in this CR downregulation by bFGF (Fig. 3, A and B).

cGMP production by OFPAE cells. The activities of pGC-A (ANP-A) and pGC-B (ANP-B) in OFPAE cells were $14.8 \pm 0.9$ (n = 4) and $0.69 \pm 0.9$ (n = 4) pmol·$10^6$ cells$^{-1}$·20 min$^{-1}$, 46.3-fold and 2.2-fold higher ($P < 0.05$) than total unstimulated production of cGMP (basal GC activity), which was $0.32 \pm 0.09$ pmol·$10^6$ cells$^{-1}$·20 min$^{-1}$ (n = 4; Fig. 4). Although we cannot eliminate the possibility of a small amount of cross reactivity of higher concentrations (1 µM) of CNP with pGC-A (ANP-A), we detected only very minor cGMP production responses during CNP treatments. Thus the vast majority of pGC in OFPAE cells is pGC-A (ANP-A) and not pGC-B (ANP-B).

By contrast, sGC activity in OFPAE cells, estimated by the treatment with a maximum stimulating dose (100 µM) of SNP, was $0.40 \pm 0.07$ pmol·$10^6$ cells$^{-1}$·20 min$^{-1}$, similar to the basal cGMP production. These data indicate that there is no detectable sGC activity in OFPAE cells (Fig. 4). Because cGMP is produced in vasculature only by sGC and pGC-A/B (ANP-A/B), these data indicate that cGMP production in OFPAE cells mostly originates from pGC-A (ANP-A).

Production of cGMP (20 min) by OFPAE cells that were maximally stimulated with 1 µM ANP after pretreatment (24 h) with 10 pg/ml–100 ng/ml bFGF was similar (Fig. 5A), indicating that bFGF did not change the maximum activities of pGC-A (ANP-A) in OFPAE cells. By contrast, 24 h of pretreatment with 10 ng/ml bFGF decreased the apparent ED$_{50}$ of ANP concentrations from 2.5 to 0.83 nM (Fig. 5B), suggesting the possibility that bFGF-mediated downregulation of CR may potentiate the OFPAE cell cGMP production response to ANP.

**DISCUSSION**

In vascular tissues cGMP is produced by both the NO-sGC and natriuretic peptides-pGC-A/B (ANP-A/B) pathways (9, 11). As illustrated in Fig. 4, treatment of OFPAE cells with ANP resulted in a 46.3-fold increase of cGMP production. By contrast, OFPAE cells had no detectable sGC activity and only a minimal 2.2-fold increase of cGMP production was observed with CNP stimulation. The latter is probably mainly due to a very low cross reactivity of CNP for pGC-A (ANP-A), although we cannot rule out the possibility of very low levels of pGC-B (ANP-B) expression (30). Thus cGMP production by OFPAE cells mostly originates from pGC-A (ANP-A). Moreover, using Western immunoblot analysis we have shown that CR protein was highly expressed in OFPAE cells (Fig. 1). Therefore, production of cGMP by OFPAE cells is regulated almost exclusively by three components, i.e., ANP concentrations, pGC-A (ANP-A) expression, and CR expression. Consequently, OFPAE cells provide a simple and convenient model to investigate the regulation and involvement of CR expression in the modulation of local cGMP production via pGC-A stimulation.

CR has been postulated to play an important role in the local removal of natriuretic peptides from the...
protein expression in OFPAE cells by bFGF was completely blocked by PD-98059, a selective MAPK inhibitor, suggesting the involvement of the MAPK signaling cascade in the downregulation of CR expression by bFGF. However, more intensive mRNA expression as well as cGMP production response studies are necessary to clarify the exact involvement of the MAPK signaling cascade. Moreover, bFGF treatment for 24 h functionally reduced the apparent ED₅₀ of ANP for stimulating cGMP production (20 min) from 2.5 to 0.83 nM. In contrast, bFGF did not change the response to maximum stimulating doses (10⁻⁶, 10⁻⁷ M) of ANP because of the saturation of both pGC-A (ANP-A) and CR by ANP (Fig. 5B). The finding that bFGF treatment had no effect on the cGMP production response with a maximal stimulating dose of ANP also suggests that the 10 ng/ml bFGF pretreatment did not change pGC-A activity in OFPAE cells. In addition, pretreatment (24 h) of OFPAE cells with 10 pg/ml–100 ng/ml bFGF did not change cGMP production (20 min) with maximal stimulation using 1 µM ANP (Fig. 5A). These data imply that bFGF increases the OFPAE cell cGMP production response to ANP treatment, probably not by upregulating maximum pGC-A (ANP-A) activity, but by downregulating CR expression and preventing removal of local ANP. More pharmacological as well as physiological studies are needed to show the exact mechanisms of these changes of cGMP production response to ANP, because we cannot rule out the possibility that bFGF may also alter the ANP receptor affinity for ANP independent of the downregulation of CR.

In summary, OFPAE cells provide a good model to investigate the involvement of CR expression in the local production of cGMP via pGC-A (ANP-A) stimulation. In addition, bFGF downregulates CR expression via the MAPK cascade, which may contribute to the increase of local cGMP production from pGC-A in OFPAE cells.

Perspectives

More detailed in vivo studies are necessary to define the physiological importance of this bFGF-induced modulation of CR in the developing placenta. Several clues, however, can be gleaned from our recent in vivo observations in which we reported that ovine placental production of bFGF is increased dramatically during the third trimester of pregnancy (34), concomitant with an increase in cGMP levels in amniotic fluid (29). Subsequently, we demonstrated in vitro that in OFPAE cells bFGF increased both NO production (unpublished data) as well as the expression of eNOS, the latter via the MAPK signal cascade (33). These data suggest that bFGF may contribute to the augmentation of cGMP production in ovine placenta vasculature during the third trimester of pregnancy. In the present study, we report that bFGF downregulated CR expression in OFPAE cells and therefore this mechanism also may contribute to increasing local fetoplacental cGMP production in vivo. Accordingly, it is likely that both the bFGF-mediated CR downregulation and the bFGF-induced eNOS upregulation and NO production contribute to increasing local cGMP production. More exten-
sive in vitro as well as in vivo studies are necessary to determine the exact physiological contribution of cGMP in the developing ovine placenta and whether these mechanisms are in part responsible for the rise in fetoplacental blood flow observed during gestation.

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