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Modulation of apelin and APJ receptor in normal and preeclampsia-complicated placentas

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Summary. Apelin is an endogenous ligand of the human orphan receptor APJ. This peptide is produced through processing from the C-terminal portion in the pre-pro-protein consisting of 77 amino acid residues and exists in multiple molecular forms. Although the main physiological functions of apelin have not yet been clarified, it is known that apelin is involved in the regulation of blood pressure, blood flow and central control of body fluid homeostasis in different organs. Since human placenta is a tissue where vasculogenesis, blood pressure and flow are dramatically important to allow a normal embryonic and fetal growth and development, the aim of the present study was to investigate the immunohistochemical distribution of apelin and APJ in normal placentas throughout pregnancy and in preeclampsia-complicated placentas. Specifically, we observed that in normal placentas the expression levels of apelin decreased from the first to the third trimester of gestation in both cytotrophoblast and syncytiotrophoblast cells and in the stroma of placental villi, in contrast with increased expression levels of APJ in the cytoplasm of cytotrophoblast cells and in the cytoplasm of endothelial cells of normal placenta samples. In contrast, in preeclampsia-complicated pregnancies, we observed a very strong increase of expression levels of both apelin and APJ receptor in all the placental compartments, cytotrophoblast, syncytiotrophoblast and stroma with a particular increase in endothelial cells inside preeclamptic placental villi. Our data seem to indicate an important role of apelin and APJ in the regulation of fetal development through a correct regulation of human placenta formation during pregnancy. Moreover, the strong expression levels of apelin and APJ in preeclamptic placentas, suggest their possible involvement in the onset of this pathology.

Key words: Apelin, APJ, Immunohistochemistry, Placenta, Preeclampsia

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Introduction

Apelin, the endogenous ligand of the human orphan receptor APJ, was firstly isolated from the bovine stomach as a peptide of 36 amino acids (Tatemoto et al., 1998). Recently, cDNA encoding apelin in mammals has been identified and characterized functionally (Tatemoto et al., 1998; Habata et al., 1999; Lee et al., 2000). It encodes 77 amino acid pre-pro-proteins which produce a biologically active form of apelin-36 from the enzymatic cleavage of C-terminus (Habata et al., 1999; Hosoya et al., 2000; Medhurst et al., 2003). APJ receptor is a 380 amino acid seven-trans-membrane domain Gi-coupled receptor that was originally isolated by PCR from human genomic DNA (O'Dowd et al., 1993). It is most closely related to the angiotensin II receptor (30-40% identity in amino acid sequence), although angiotensin II does not interact with the APJ receptor when expressed in Chinese hamster ovary (CHO) cells (Tatemoto et al., 1998) or in fibroblasts (O'Dowd et al., 1993). Apelin and APJ mRNAs and proteins were detected in different human and vertebrate tissues as well as stomach, brain, heart, lung, blood vessels and many others (Tatemoto et al., 1998; Habata et al., 1999; De Mota et al., 2000; Hosoya et al., 2000; Lee et al., 2000; O'Carroll et al., 2000; Katugampola et al., 2001; Kawamata et al., 2001; Brailoiu et al., 2002; Reaux et al., 2002; Cheng et al., 2003; De Falco et al., 2002, 2004). Several studies have been suggesting an apelin cardiovascular role due to the presence of the peptide and APJ receptor in heart and blood vessels. In addition, it has been demonstrated that intravenous injection of apelin decreased blood pressure in anaesthetized rats (Lee et al., 2000). Moreover, it has

been demonstrated that apelin is able to modulate contractility of cardiac tissue and blood vessels (Katugampola et al., 2001; Skokodi et al., 2002), pituitary hormone release (Reaux et al., 2001), fluid consumption (Lee et al., 2000; Reaux et al., 2001; Taheri et al., 2002), cytokine suppression (Habata et al., 1999). Despite a large number of reports describing expression and distribution of apelin and its receptor APJ in different organs and tissues, little is known about their physiological roles. All the apelin, angiotensin II and bradykinin peptides have blood pressure-modulating effects, are expressed in cardiovascular tissue and are cleaved by human endothelial angiotensin I-converting enzymes (Vickers et al., 2002). The placenta is a tissue where vasculogenesis and blood pressure and flow are dramatically important to allow a normal fetal development and growth. Alterations of these elements cause a typical pregnancy pathology: the preeclampsia. The current clinical definition of preeclampsia is hypertension normally occurring after the 20th week of gestation and accompanied by proteinuria (Page, 2002). Physiologically, the symptoms of preeclampsia are unanimously viewed as being caused by multi-system dysfunction, and the complex interaction and imbalance of many endocrinological mechanisms has been reported as a major factor contributing to the widespread pathology of preeclampsia (Page, 2002). However, although the exact patho-physiological mechanism of preeclampsia is not clearly understood, it can be considered as a disorder of endothelial function with vasospasm. Due to recent literature about apelin/APJ roles in blood homeostasis, we have decided to investigate their expression and cellular localization in human placenta during physiological (throughout the gestation) and preeclampsia-complicated pregnancies.

Materials and methods

Samples

Sample category consists of normotensive pregnant patients and pregnant patients with preeclampsia (an increase in systolic blood pressure of 30 mm Hg, an increase in diastolic blood pressure of 15 mm Hg, or blood pressure greater than 140 mm Hg/90 mmHg as absolute reading, a significant increase of proteinuria and of weight) (Table 1).

Placentas from the first trimester of gestation were obtained from uterine evacuations (n=15) and samples from the third trimester of gestation were immediately obtained from cesarean sections (n=15). Samples of pregnant patients with preeclampsia were obtained from cesarean delivery (n=15). All the samples were obtained with informed consent of patients. The gestation age of the 1st trimester samples ranged from 5 to 14 weeks and the gestational age of 3rd trimester specimens ranged from 28 to 40 weeks. The gestational age of preeclamptic specimens ranged from 35 to 40 weeks. All the collected specimens were immediately fixed in formalin (n=45; 15 from each trimester of gestation and 15 from preeclampsia complicated pregnancy) for immunohistochemistry. Representative sections of each specimen were stained with haematoxylin-eosin and examined by a pathologist to confirm histological preservation of the microanatomical structure for normal samples. Protocols involving human tissues in this research were approved by our institutional human research studies committee.

Immunohistochemistry

Immunohistochemistry was carried out essentially as described previously (De Falco et al., 2004). Briefly, sections from each specimen were embedded in paraffin, cut at 3-4 µm, mounted on glass and dried overnight at 37°C. All sections then were deparaffinized in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide and blocked with PBS-6% milk for 1 hr at room temperature. Slides then were incubated at 4°C overnight with the rabbit antibody raised against apelin-17 (kind gift by Dr. Tatemoto) at a 1:500 dilution and with the mouse monoclonal antibody raised against APJ (kind gift of Dr. Medhurst) at 1:62 dilution. After several washes to remove the excess of antibody, the slides were incubated with diluted goat anti-rabbit or goat anti-mouse biotinylated antibodies (Vector Laboratories) for 1 h. All the slides then were processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Novared (Vector Laboratories) was used as the final chromogen. Negative controls for each tissue section were prepared by substituting the primary antiserum with the isotype-matched non-immune rabbit and mouse IgG. For each experiment, all slides were stained in a single batch and thus received equal staining. The expression level of apelin- and APJ-stained cells per field at light microscopy, at a 20 X original magnification, was calculated and compared in different specimens by three separate observers and described as: score 0 (absent); score 1 (weak); score 2 (moderate); score 3 (intense), as described by Seelam et al. (2001).

Table 1. Sample features of pregnant patients.

Characteristics	Normotensive n = 15	Mild preeclampsia n = 15
Age (year)	25±0.6	30±2
Weight (Kg)	65±2	78±2
Hemoglobin level (g/dl)	12.2±1	12.2±1.2
Hematocrit (%)	38.9±2	37±0.8
Systolic blood pressure (mm Hg)	115±6	145±4
Diastolic blood pressure (mm Hg)	76±4	92±2
Proteinuria (mg in 24 h)		<300
Gestational age (weeks)	39	37

For each specimen, an HSCORE value was derived by summing the percentages of cells/areas that stained at each intensity and multiplying that by the weighted intensity of the staining. For example:

$$HSCORE = \Sigma\ Pi\ (i + 1)$$

where *i* represents the intensity scores and Pi the corresponding percentage of cells/areas.

An average of 22 fields was observed for each tissue by three observers at different times and the average score was used. All values were expressed as mean ± standard error of mean (SEM) and differences were compared using Student's *t*-test.

Results

Localization and pattern of expression of apelin in human placenta during normal and preeclampsia-complicated pregnancy

We investigated the expression of apelin in normal and preeclampsia-complicated human placentas throughout the gestation by immunohistochemistry. We observed that in the first trimester of gestation, apelin was localized at moderate/intense levels of expression in the cytoplasm of cytotrophoblast cells forming the inner proliferative layer of placental villi (Fig. 1a). Moreover, an intermediate immunoreactivity for apelin was also observed in the stroma of placental villi (Fig. 1b), in contrast with a weak immunopositivity in syncytiotrophoblast cells (Fig. 1a,b). During the third trimester of gestation, apelin expression levels decreased, with a positivity localized almost exclusively in the cytoplasm of cytotrophoblast cells (Fig. 1c,d). A faint immunoreactivity was detectable in syncytiotrophoblast cells and in the stroma of placental villi.

In preeclamptic placentas, we observed a strong increase of apelin expression levels. Specifically, we found a strong apelin immunopositivity in the cytoplasm of both cytotrophoblast and syncytiotrophoblast cells, together with a diffuse immunopositivity in the stroma of placental villi (Fig. 1e).

No immunostaining was observed in the negative controls performed by substituting the primary antiserum with the isotype-matched non-immune rabbit IgG (Fig. 1f).

Localization and pattern of expression of APJ in human placenta during normal and preeclampsia-complicated pregnancy

As regards APJ localization, we observed that, in the first trimester of gestation, this receptor was localized at moderate/intense levels of expression mostly in the cytotrophoblast cells (Fig. 2a). In particular APJ was distributed both in the cytoplasm and the nucleus of cytotrophoblast cells (Fig. 2b). A weak immunopositivity for APJ was found in syncytiotrophoblast cells and in the stroma of placental villi during the first trimester of gestation (Fig. 2 a,b). In the third trimester of gestation, the expression levels of APJ strongly increased mainly in the cytotrophoblast cells (Fig. 2 c), with a localization in the cytoplasm and the nucleus (Fig. 2d). Moreover, very strong APJ expression levels were observed in the cytoplasm of endothelial cells inside placental stroma (Fig. 2c), in contrast with always weak immunopositivity in syncytiotrophoblast cells (Fig. 2 d).

In preeclamptic placentas, we observed that expression levels for APJ strongly increased too. In particular, we observed a strong immunopositivity for the receptor in all the placental compartments, cytotrophoblast, syncytiotrophoblast cells and stroma, with a subcellular localization both at cytoplasmic and nuclear level (Fig. 2e). In addition, we found very intense expression levels for APJ in the cytoplasm and the nucleus of endothelial cells inside stroma of preeclamptic placental villi (Fig. 2f).

No immunostaining was observed in the negative controls performed on normal placenta slides of the first and the third trimester of gestation, by substituting the primary antiserum with the isotype-matched non-immune mouse IgG (Fig. 2g,h).

In figure 3 the whole expression pattern of apelin and APJ during normal and preeclampsia-complicated pregnancies, as detected by immunohistochemical assay, is depicted. Specifically, we observed a marked decrease of apelin expression levels from the first to the third trimester of gestation in cytotrophoblast cells and in the stroma of normal placentas together with a slight decrease in the syncytiotrophoblast compartment in the third trimester of gestation. On the contrary, in preeclamptic placentas we observed increased expression levels for apelin in all the placental compartments, cytotrophoblast, syncytiotrophoblast and stroma. Moreover, we observed an increase of APJ expression levels from the first to the third trimester of gestation, mainly in the cytoplasm of cytotrophoblast cells and endothelial cells surrounding placental capillaries inside normal placental villi. In preeclamptic placentas, instead, we found a very strong increase of APJ expression levels in cytotrophoblast, syncytiotrophoblast, stroma and endothelial cells.

Discussion

Apelin has a widespread pattern of expression in human tissues (De Falco et al., 2002) and it is produced in several tissues of the body, including brain, lung, pregnant and lactating breast, and GI tract (Habata et al., 1999; O'Carroll et al., 2000). Apelin is also expressed in the vascular endothelial cells lining blood vessels of the human heart (Kleinz and Davenport, 2004) and rat blood vessels (Tatemoto et al., 2001). Moreover, apelin mRNA was abundantly expressed in cultured human endothelial cells (Medhurst et al., 2003). APJ localizes in a wide variety of tissues, the endothelial cells of the primary blood vessels, and in forming heart (Devic et al., 1999), in the pulmonary system (Habata et al., 1999) and in the spleen (Edinger et al., 1998). Like many other regulatory

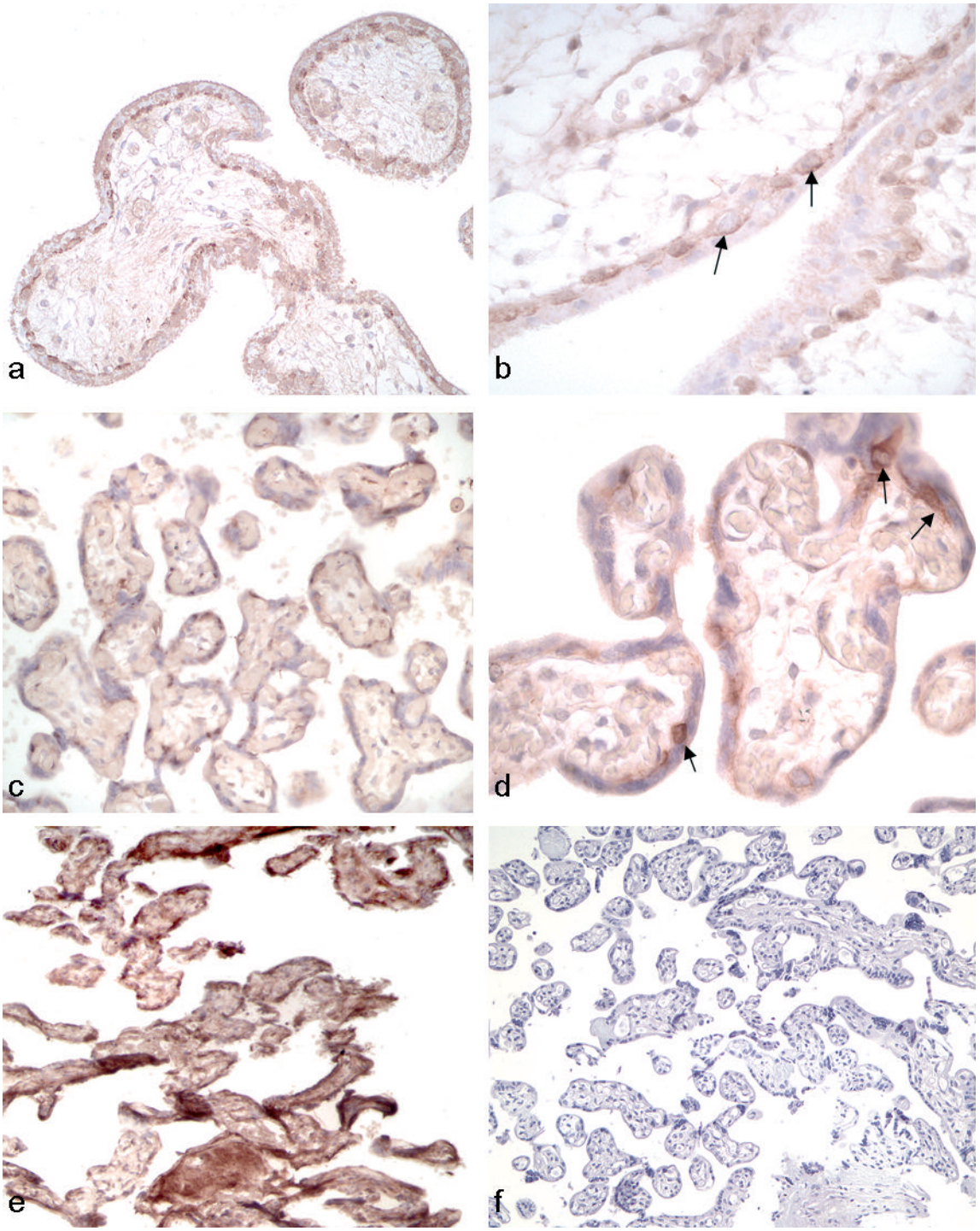

Fig. 1. Localization of apelin in normal and preeclamptic human placentas throughout gestation. **a.** Apelin localization in the cytoplasm of cytotrophoblast cells during the first trimester of gestation of normal placenta. x 150. **b.** Higher magnification showing the cytoplasmic immunostaining (arrows) of apelin in cytotrophoblast cells of first trimester normal placental villi. x 450. **c.** Apelin decreased expression levels in the cytotrophoblast cells in the third trimester of gestation of normal placenta. x 150. **d.** Higher magnification showing the immunoreactivity for apelin inside the cytoplasm of cytotrophoblast cells (arrows) of third trimester normal placental villi. x 450. **e.** Apelin intense immunopositivity in the cytoplasm of both cytotrophoblast and syncytiotrophoblast cells of preeclamptic placental villi. x 150. **f.** Absent immunostaining in a representative negative control of preeclamptic placenta. x 100

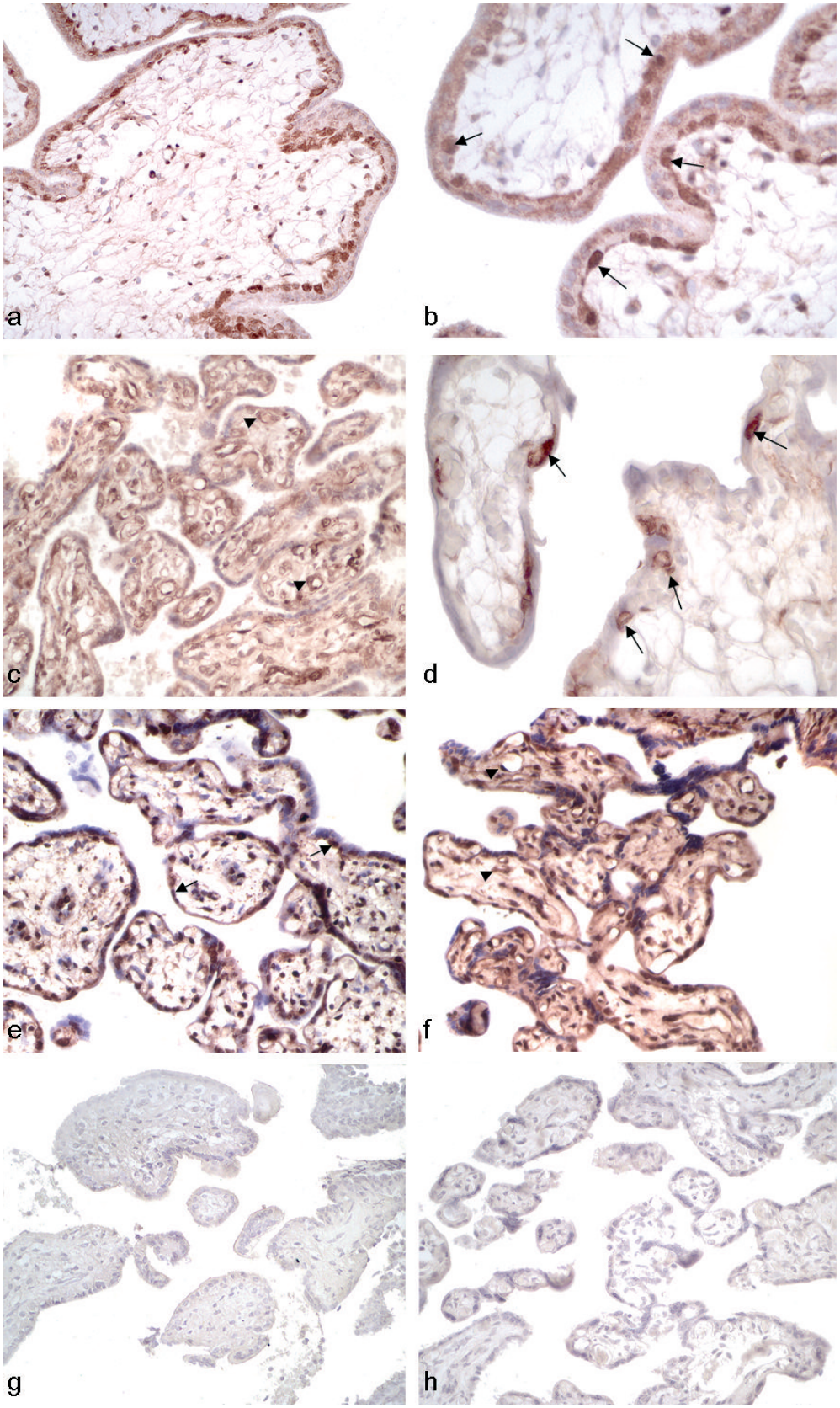

Fig. 2. Localization of APJ in normal and preeclamptic human placentas throughout gestation. **a.** APJ localization in cytotrophoblast cells in the first trimester of gestation of normal placenta. x 150. **b.** Higher magnification showing the subcellular localization of APJ in the cytoplasms and the nuclei of cytotrophoblast cells (arrows). x 450. **c.** Intense immunostaining for APJ in the cytotrophoblast cells and in endothelial cells (arrow heads) inside the stroma of normal placental villi in the third trimester of gestation. x 150. **d.** Higher magnification showing the immunoreactivity for APJ in the cytoplasms and in the nuclei of cytotrophoblast cells (arrows) in normal third trimester placentas. x 450. **e.** APJ strong immunopositivity in cytotrophoblast cells and syncytiotrophoblast cells at cytoplasmic and nuclear (arrows) levels in preeclamptic placental villi. x 150. **f.** Very intense immunostaining for APJ localized endothelial cells (arrow heads) inside the stroma of preeclamptic placental villi. x 200. **g.** Representative negative control of a normal placenta of the first trimester of gestation. x 150. **h.** Representative negative control of a normal placenta of the third trimester of gestation. x 150

peptides, pharmacological studies indicate that apelin and APJ have multiple biological activities. Reported actions for apelinergic system include inhibition of pro-inflammatory cytokine production by mouse spleen cells (Habata et al., 1999), chemotactic activity on CHO-A10 cells (Hosoya et al., 2000), lowering of blood pressure (Lee et al., 2000) and stimulation of drinking behaviour in rats (Reaux et al., 2001). In the present study, we first investigated the pattern of expression of apelin and APJ receptor in human placenta during whole gestation, demonstrating that there is a modulation of apelinergic system throughout pregnancy. Specifically, we found that apelin expression levels slightly decreased from the first to the third trimester of gestation, whereas APJ immunoreactivity strongly increased in the third trimester of gestation. Moreover, we found that

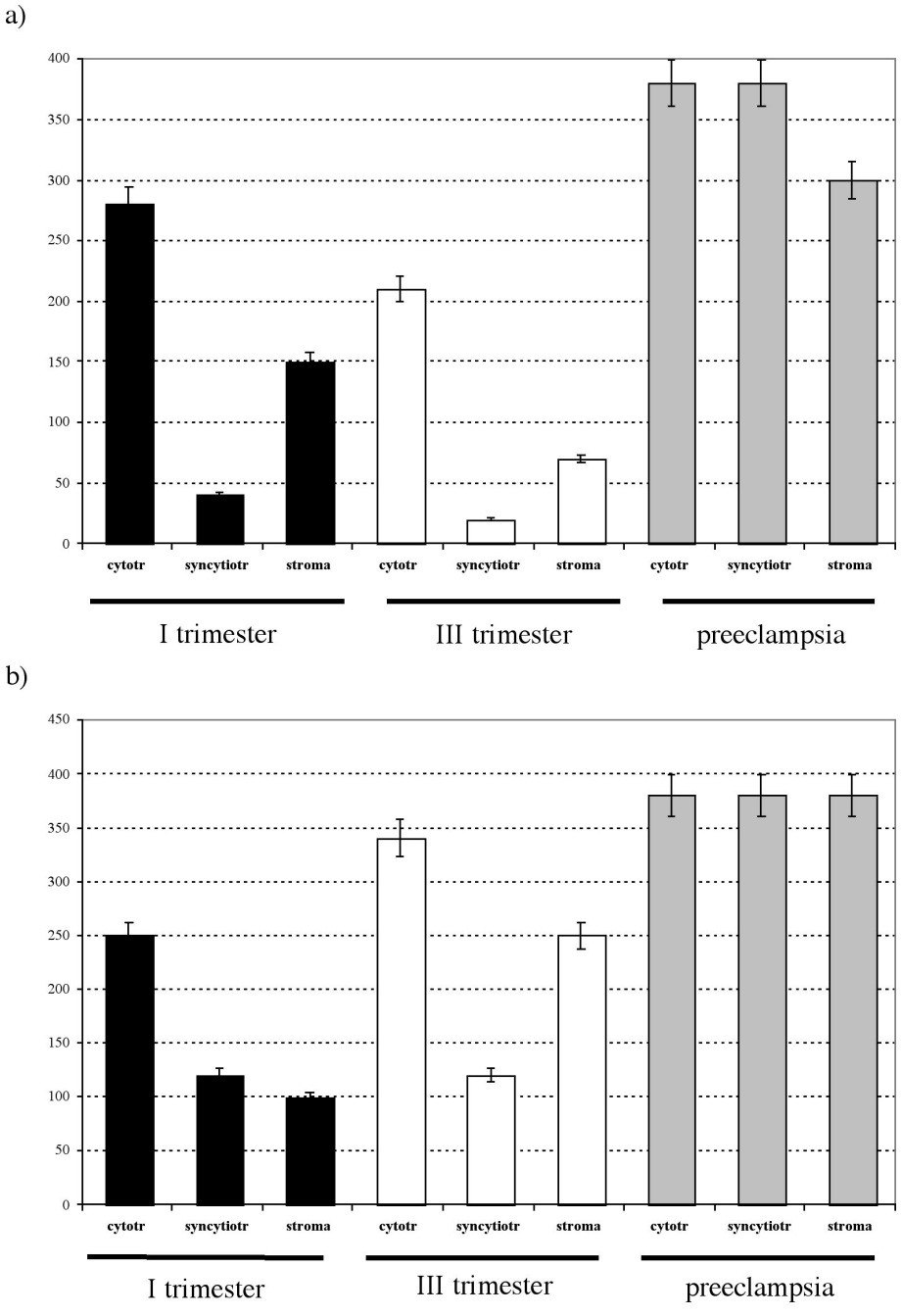

Fig. 3. Gestational time course of cytotrophoblast, syncytiotrophoblast and stromal cell immunoreactivity for apelin and APJ in normal and preeclampsia-complicated pregnancy. **a.** Expression level of apelin in placental villi of the first and the trimester of gestation, and in preeclamptic placentas. Staining intensity for apelin was significantly higher in the preeclamptic placentas compared with each trimester of gestation of normal placentas ($P<0.05$). **b.** Expression level of apelin in placental villi of the first and the trimester of gestation, and in preeclamptic placentas. Staining intensity for APJ was significantly higher in the preeclamptic placentas compared with each trimester of gestation of normal placentas ($P<0.05$) Ordinate: immunoreactivity scored as described in materials and methods. Abscissa: Placental compartments. Vertical lines show S.E.M.

immunopositivity for apelin and APJ was mostly localized in cytotrophoblast cells during both the first and the third trimester of gestation. Since the cytotrophoblast cells represent the proliferative population inside placental villi, this particular localization might suggest a possible role of the apelinergic system in the control of proliferation, as recently demonstrated on gastric epithelial cells (Wang et al., 2004) and on umbilical endothelial cells (Masri et al., 2005). In addition, we also described a nuclear localization of APJ receptor in many cytotrophoblast and endothelial cells inside placental villi. These data are in concordance with previous data by Lee et al. (2004), indicating the nuclear localization of APJ receptor in human brain and suggesting an important role of this receptor in cell signaling and function (Lee et al., 2004). Among multiple supposed roles of apelinergic system, many studies have been focused on its involvement in blood pressure. In particular, it has been demonstrated that apelin is expressed in the endothelial layer of the retinal vessels (Saint-Geniez et al., 2002) and at high level in endothelial cells of other vessels (De Falco et al., 2002; Kleinz and Davenport, 2004). In the light of these observations, apelin has been proposed as a local vasoactive mediator (Kleinz and Davenport, 2004). Starting from this hypothesis, we thought to investigate the pattern of expression of apelin and APJ receptor in preeclampsia-complicated pregnancies, demonstrating a strong increase of expression levels of both apelin and APJ in all the placental compartments, cytotrophoblast, syncytiotrophoblast, endothelial cells and stroma of placental villi. Preeclampsia is a highly variable and dangerous complication of the second half of pregnancy (Redman and Sargent, 2003). In particular, in 1989 Roberts first suggested that endothelial dysfunction may occur in preeclampsia, proposing that one of the specific targets was the maternal vascular endothelium (Roberts et al., 1989). Subsequently, it has been demonstrated that the levels of nitrate and nitrite, major metabolites of NO, a key player in endothelial dysfunctions, decreased in the serum and urine of preeclampsia women (Seligman et al., 1994; Davidge et al., 1996). It has been postulated that apelin expressed by endothelial cells, acting on vascular smooth muscle in a paracrine fashion, may have a vasoconstrictor potential. In the presence of a functional endothelium, instead, this vasoconstrictor effect may be counterbalanced through the release of endothelial vasodilator substances, such as nitric oxide (NO) (Kleinz and Davenport, 2004). Since it has been demonstrated that in preeclampsia the dysfunction of the NO system leads to a deficiency of nitric oxide (Seligman et al., 1994; Davidge et al., 1996), we can hypothesize that the strong increase of apelin and APJ expression levels observed in preeclamptic placentas, might induce a vasoconstrictor effect determining the onset and/or worsening of this placental pathology. These data appear quite interesting to elucidate the pathophysiologic mechanism of this disorder and to identify specific biochemical markers in order to predict the early onset of preeclampsia.

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