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Expression of OP4 (ORL1, NOP_1) receptors in vascular endothelium

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Abstract

Endothelial cells from rat brain microvessels, human aortic artery and human umbilical vein were examined, together with ex vivo rat brain capillaries and rat aortic ring sections, for the expression of opioid receptor-like OP-4 mRNA and protein. High levels of mRNA expression and an immunopositive reaction for the receptor protein were detected in the endothelial cells from primary and from established in vitro cultures, as well as in the intima of ex vivo rat aortic rings, where the signal was limited to the endothelial layer. Interaction of the OP4 receptor with its physiological ligand nociceptin caused, in cultured endothelial cells, the activation of a mitogen-activated protein (MAP) kinase cascade. Taken together, these results show that the OP4 receptor is synthesised and functionally expressed in endothelial cells, presumably as a starting point for some vasoactive mechanism(s).

Keywords: Endothelial cell; Aorta; OP4 receptor; Immunocytochemistry; In situ hybridization; MAP (mitogen-activated protein) kinase

1. Introduction

A new member has been added to the family of the three major opioid receptors by the discovery in humans (Mollereau et al., 1994) of a fourth opioid-like receptor, OP4, which was originally referred to as ORL1 (opioid receptor-like1) and which is now indicated, after the identification of its endogenous ligand nociceptin, referred to as NOP_1 (Mogil and Pasternak, 2001). The OP4 receptor is most abundant in the central nervous system (Meunier et al., 1995) and in peripheral nervous tissue (Calò et al., 2000). It has however been found also in other peripheral tissues of the rat, namely in intestine and vas deferens (Menzies et al., 1999), as well as in human blood lymphocytes (Wick et al., 1995). OP4 receptor transcripts have also been detected, by a reverse transcriptase-polymerase chain reaction (RT-PCR), in several peripheral sensory and sympathetic ganglia from guinea-pigs (Kummer and Fischer, 1997) and rats (Xie et al., 1999), while high-affinity binding sites for nociceptin have been described in rabbit retina (Neal et al., 1997) and in rat heart (Giuliani et al., 1997; Dumont and Lemaire, 1998). The nociceptin/OP4 receptor system presumably subserves, therefore, a number of important physiological functions (Calò et al., 2000). Since OP4 receptors are reportedly present on pre- and/or postganglionic sympathetic and parasympathetic nerve fibres innervating blood vessels and heart and/or directly on these target organs, they are most probably involved in the cardiovascular effects of nociceptin, and play therefore a role in the pathophysiology of inflammation, arterial hypertension and cardiac or brain circulatory ischaemia (Malinowska et al., 2002). The

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concentration-dependent vasorelaxation responses to nociceptin, observed in arterial segments precontracted by phenylephrine, have however been shown to occur only if the endothelium was also present (Hugghins et al., 2000).

The OP4 receptor greatly resembles the opioid receptor in terms of signal transduction properties. It is coupled to some G_i/G_o protein(s), and mediates the intracellular effects of nociceptin, such as inhibition of adenylyl cyclase activity, closure of voltage-sensitive Ca^{2+} channels, increase in K^+ conductance (Calò et al., 2000). Stimulation of the OP4 receptor has been shown to lead, via a specific mitogen-activated protein (MAP) kinase pathway (Lou et al., 1998), to the phosphorylation of extracellular signal regulated kinase(s) (ERK1 and/or ERK2).

The purpose of this study was to investigate the expression and the functional activity of the OP4 receptor in endothelial cells from various vascular regions.

2. Materials and methods

2.1. Tissues and cells preparations

Thoracic aorta tissue and primary cultures of brain microvessel endothelial cells were prepared from male Sprague–Dawley rats (250–300 g). All animals were killed according to the recommendations of the European Veterinary Medical Association, as required by the Ministry of Health. Segments of thoracic aorta were cleaned of adhering perivascular tissue and embedded in paraffin to preserve the integrity of the endothelium.

Rat brain microvessels were prepared from the cerebral cortex by a standard mechanical procedure (Cardelli et al., 2001), and were either used as such or as a source of endothelial cells from this particular vascular region. To this purpose, the isolated capillary fragments were dissociated and purified by centrifugation in a 25% (w/v) bovine serum albumin solution. The pellet was then resuspended in a collagenase/dispase solution and centrifuged, according to Abbott et al. (1992), on a 50% Percoll isotonic gradient, thus obtaining an endothelial cell suspension that was distributed in Petri dishes maintained in Ham's F10 medium supplemented with 15% Foetal Calf Serum. The primary culture was subcultured only once and used in the experiments. These populations of brain microvessel endothelial cells were routinely characterised by immunodetection of specific endothelial markers.

Commercial human aortic artery cells (purchased from Bio Whittaker) and human umbilical vein endothelial cells were also used.

2.2. In situ hybridization

Full-length rat and human OP4 c-DNAs were amplified by PCR by using primers based on the reported human or rat sequences (Meunier et al., 1995). The fragments were subcloned in pcDNA3 (Invitrogen Life Technologies). The Sp6 and T7 RNA polymerases were used to synthesise, by using a polymerase labelled kit (Rote Diagnostics), digoxigenin-labelled antisense and sense probes. The in situ hybridization was performed overnight at 40 °C with aorta ring sections (5 μm) and with cultured cells, according to Herrington and Mc Gee's (1992) protocols, in the presence of digoxigenin-labelled probes (0.1 ng/μl) diluted in the hybridization solution (20 mM Tris–HCl, 1 mM EDTA, 300 mM NaCl, 50% formamide, 10% dextran sulphate, 1 × Denhardt's solution, 250 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, 0.1% sodium dodecyl sulphate and 0.1% sodium thiosulphate).

Following several washes with 100 mM Tris–HCl/0.9% NaCl (pH 7.5) containing 3% normal goat serum and 0.3% Triton X-100, the specimens were incubated with alkaline-phosphatase-labelled anti-digoxigenin Fab fragment (1:1000), with a final colorimetric detection step consisting of a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate system. Controls included hybridization in the presence of labelled probes in the sense orientation.

2.3. Production of rabbit polyclonal antibody

The expression of OP4 receptor proteins was also studied by a Western blot procedure, using an antibody prepared in our laboratories. The antibody against the OP4 receptor was raised against a synthetic MESLFPAPFWEVLYGSHF peptide, corresponding to the 1–18 N-terminal amino acid sequence of the OP4 receptor, selected through a Blastmail Genebank software program according to the following parameters: highly conserved sequence among species, high divergence from the protein sequence of known opiate receptors and no significant homology with other proteins.

A lysine (K) at position 19 was added in order to allow the covalent coupling of the antigen to cationic bovine serum albumin (''Super Carrier Immune Modulator'', Pierce). The peptide (20 mg) was dissolved in 2 ml of a carrier solution (0.1 M carbonate buffer pH 8.5 and 1% glutaraldehyde) and purified on a G-25 Sephadex gel chromatography column equilibrated with 0.1 M phosphate-buffered saline, pH 7.4. The fractions were used as immunogens. Antibodies were raised in rabbits by injecting 500 μg of immunogen with successive injections of 300 μg each at 10-day intervals. Blood was collected 5 days after each immunisation and tested for immunofluorescence.

The specificity of the polyclonal primary antibody was also checked by using an African Green Monkey kidney SV40-transformed cell line (COS-7) that had been transiently transfected with cDNA encoding the OP4 receptor. Immunostaining was observed in the transfected COS-7 cells, while no labelling was reported in the non-transfected cells (data not shown).

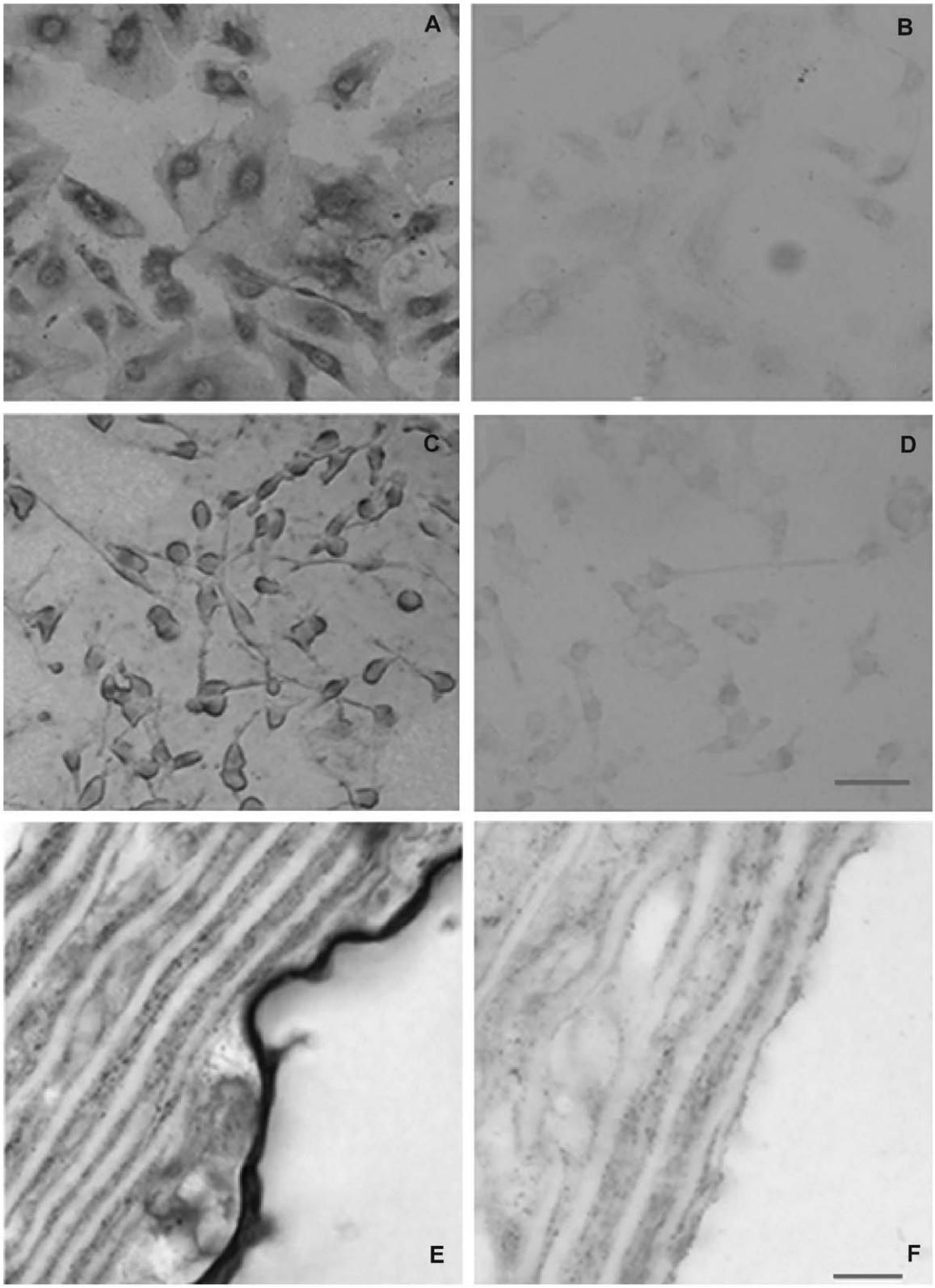

Fig. 1. Representative brightfield photomicrographs showing OP4 receptor mRNA expression in a human aortic artery endothelial cell line (A, B), in cultured endothelial cells from rat brain microvessels (C, D), and in a ring section of ex vivo rat aorta. In the isolated cells, the positive mRNA signals obtained using a digoxigenin-labelled antisense cRNA probe exhibited granular reactivity concentrated mainly in the cytoplasm and particularly in the perinuclear area, as well as in the typical elongated processes (A and C). The specificity of the hybridization is evidenced by the absence of specific labelling when using a sense probe as a negative control (B and D). In the rat aortic ring section, a positive in situ hybridization reaction was obtained, with the digoxigenin-labelled OP4 antisense probe (E), only in the endothelial layer, while it was absent from the adjacent median layer and from the adventitia. No reaction was obtained with the sense probe (F). The scale bar corresponds to 35 μm in panels A, B, C and D, to 25 μm in E and F.

2.4. Immunocytochemistry

Anti-OP4 receptor immunoreactivity was measured in fixed endothelial cells from either brain microvessels, human aorta or human umbilical vein, as well as in aorta ring sections, by using streptavidin–biotin–peroxidase or indirect immunofluorescence. The immune reaction was revealed by using either an anti-rabbit immunoglobulin G (IgG)-biotinylated antibody and the streptavidin–biotin–peroxidase complex (Amersham, UK), followed by incuba-

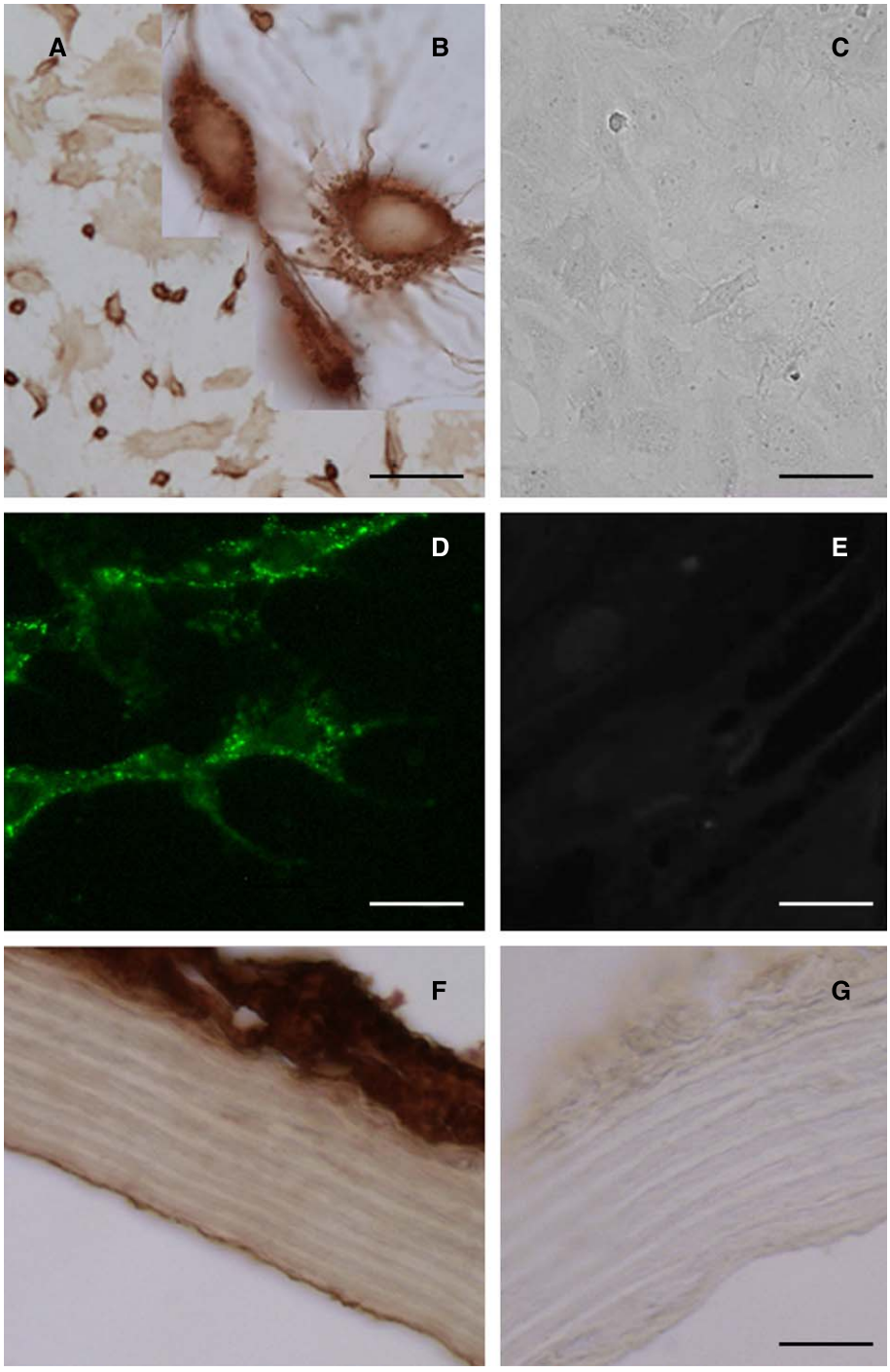

Fig. 2. Representative patterns of different immunohistochemical reactions for OP4 immunodetection. In human aortic artery endothelial cells, streptavidin–biotin–peroxidase immunostaining shows a positive immunoreaction which is particularly concentrated in the perinuclear area, with a markedly positive granular pattern (A and, at higher magnification, insert B). In rat brain endothelial cells (D), the indirect fluorescent immunoassay shows fairly homogeneous binding of the primary antibody, underlying several punctuate fluorescent marked spots in both soma and extension of the cells, thereby showing the typical elongated endothelial morphology. In a ring section of rat aorta, streptavidin–biotin–peroxidase immunostaining is highly concentrated in the vascular endothelium layer (F). All controls, performed with non-immune serum, were negative (C, E, G). Scale bar: 25 μm.

tion with H_2O_2/diaminobenzidine (10 μl and 3 mg, respectively, in 10 ml of phosphate-buffered saline), or an anti-rabbit IgG antibody conjugated with fluorescein isothiocyanate (Santa Cruz, USA). The specimens were observed under a white light or a fluorescence microscope (Leitz, Germany). Negative controls were performed by replacing the primary antibody with rabbit non-immune IgG.

2.5. Western blot analysis

OP4 receptor immunoreactivity was measured in human umbilical vein endothelial cells and in brain capillaries, using rat brain hippocampus and spleen homogenates as positive and negative controls. All preparations were homogenised in ice-cold sodium dodecyl sulphate (SDS)-containing lysis buffer at 4 °C, resuspended in SDS-bromophenol blue reducing buffer and routinely tested on Coomassie-stained SDS polyacrylamide gels for a quality estimate of the extracts. The lysates (100 μg protein) were separated by electrophoresis on a 8% SDS-polyacrylamide gel and blotted on polyvinylidene difluoride membranes, which were then incubated overnight at 4 °C with the anti-OP4 primary antibody (1:600). The immunoreactive bands were visualised by using the enhanced chemiluminescence (ECL) Western blotting kit (Amersham). The intensity of these bands, evaluated by semiquantitative image analysis performed with Scion Image software (NIH, Bethesda) and with reference to the intensity of the β-actin band from the same sample, was compared by ANOVA followed by Fisher's test of probability at the least significant differences.

2.6. Mitogen-activated protein (MAP) kinase assay

The day before the MAP kinase assay, cultured human umbilical vein endothelial cells were transferred to a serum-free medium, in order to obtain the basal level of MAP kinase phosphorylation. Nociceptin (1 μM) was then added to the cells and allowed to incubate for either 5 or 20 min at 37 °C. The cells were then rapidly rinsed with ice-cold PBS and solubilised for 15 min in Triton X-100 lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 10 mM glycerophosphate), being thereafter cleared by centrifugation (10 min at 10,000 × *g*). The lysates (80 to 100 μg protein) were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes and probed using a 1:2000 dilution of a commercial anti-phosphospecific antibody (New England Biolabs) raised against phosphorylated MAP kinase. The phosphorylation of MAP kinase by 10 mM phorbol 12-myristate,13-acetate was used as positive control. The immunoreactive bands were visualised using a highly sensitive horseradish peroxidase ECL-based Western blotting kit (Amersham).

3. Results

High levels of mRNA expression for the OP4 receptor were found in all the endothelial cells studied. In endothelial cells derived from human aorta or from rat brain microvessels, the specific mRNA signal was evidenced by granular reactivity, mainly concentrated in the perinuclear area (Fig. 1 A,C). Similarly high levels of OP4 mRNA expression were observed in the endothelial layer of the rat aorta ring sections (Fig. 1E). The signal was found to be specific for the endothelium, since no mRNA signal could be detected in the adventitia or in the median layer. No labelling was observed either in the cells or in the sections hybridized with the sense probe (Fig. 1B,D,F).

Positive patterns of immunoreactivity for the OP4 receptor protein were also detected in the various endothelial cells, as demonstrated by either the indirect immunofluorescence or the streptavidin–biotin–peroxidase immunochemical procedure. The endothelial cells derived from human aorta or from rat brain microvessels exhibited a large number of punctuated spots both in the cell body and in the peripheral extensions, showing a typical elongated endothelial morphology (Fig. 2A,B,D). In rat aorta ring sections, the protein distribution pattern, studied by streptavidin–biotin–peroxidase immunostaining, was similar to that of the mRNA signal, being predominantly distributed in the vascular endothelial layer (Fig. 2F).

Western blot analysis of endothelial cells from human umbilical vein or from rat brain capillaries was performed for a better evaluation of the OP4 receptor protein signal intensity in the endothelium, as compared to the brain regions where the OP4 receptor is reportedly present. Hippocampus and rat spleen were chosen as positive and negative controls, respectively. A major 64-kDa band that reacted with the anti-OP4 receptor antibody was detected in both cell types (Fig. 3). A semiquantitative analysis of the band intensities indicated that the level of expression of the OP4 receptor, when normalized to that of a housekeeping gene product such as β-actin, was

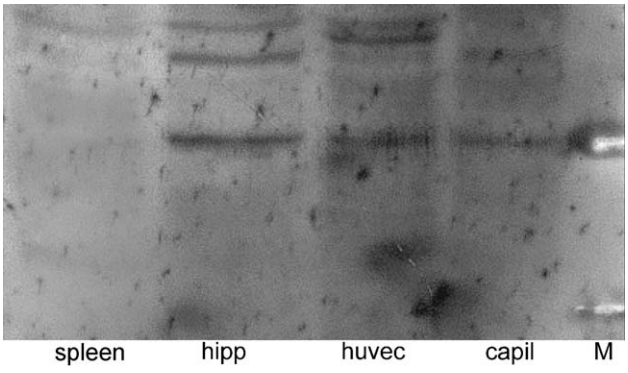

Fig. 3. Immunoblot showing OP4 receptor expression, as a 64-kDa band, in the whole rat hippocampus (hipp), in human umbilical vein endothelial cells (huvec) and in rat brain capillaries (capil). No OP4 receptor expression was evidenced in rat spleen. M = marker.

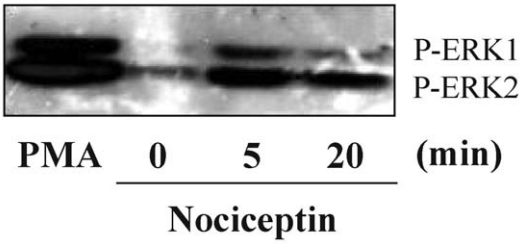

Fig. 4. Induction by nociceptin of ERK phosphorylation in human umbilical vein endothelial cells. Quiescent cells under basal conditions were exposed to nociceptin (1 μM) for 5 and 20 min. The phosphorylated derivatives (P-ERK1 and P-ERK2) of the ERK isoforms were detected by immunoblotting with an anti-phosphospecific antibody. Exposure to PMA (10 nM) afforded a positive control.

rather similar in the endothelial cells from human umbilical vein or rat brain capillaries, while the intensity of the same band was around 60% higher in extracts from rat hippocampus.

In order to establish the efficacy of the studied receptor in activating its specific signal transduction pathway, we also examined the OP4 receptor-mediated selective activation of ERK1 and/or ERK2. Upon addition of 1 μM nociceptin to human umbilical vein endothelial cells, the phosphorylated (“activated”) forms of both ERK1 and ERK2 increased well above the non-stimulated control levels. This effect occurred quite rapidly, i.e. within 5 min, and exhibited a significant, almost parallel, decrease after 20 min (Fig. 4).

4. Discussion

To our knowledge, we present the first reported evidence of OP4 mRNA and protein expression in the endothelium and, in particular, of a specific distribution of OP4 receptors in the endothelial lining of aortic vessels. These data are in line with some previous reports that indicate a critical role of endothelium in mediating the vasoactive effects of nociceptin. Nociceptin is known to have a direct vasorelaxant action on peripheral arterial blood vessels (Champion et al., 1998), but removal of the endothelial layer has been shown to cause a loss of nociceptin-induced vasodilatation in large blood vessels, as well as a loss of the ability to decrease the isometric tension of phenylephrine contracted aortic rings (Hugghins et al., 2000). According to Lin et al. (2000), nociceptin-induced systemic hypotension could occur through a specific nociceptin/OP4 receptor system coupled to the release of nitric oxide, which mediates vasodilatation, in the systemic vascular bed. We have shown that functional OP4 receptors are present in the endothelial lining of small and large blood vessels and in the endothelial cells of brain microvessels. The OP4 receptors appear therefore to link the effects of nociceptin in the central nervous compartment to those found in blood vessels, including the blood–brain barrier.

Brain capillaries and microvascular endothelial cells are indeed known to express receptors for several neurotransmitters, including dopamine (Scriba and Borchardt, 1989), noradrenaline (Bacic et al., 1992), acetylcholine (Traish et al., 1994), opioids (Stefano et al., 1995), and serotonin (Bouchelet et al., 1996), for hormones such as somatostatin (Cardelli et al., 2001), for endothelins (Masaki et al., 1999), and for vasoactive mediators such as angiotensin (Guillot and Audus, 1991), histamine and adenosine (Stanimirovic et al., 1994). These effectors have been shown to modulate vascular tone (Luscher, 1990) and/or blood–brain barrier permeability by acting on the endothelial receptors (Guillot and Audus, 1991).

Since OP4 receptor mRNA and the corresponding protein were found in all types of endothelial cells tested, and in particular in the endothelial lining of brain microvessels, we suggest that the synthesis and functional expression of the OP4 receptor on brain endothelial cells might contribute to modulation of the vascular tone of brain microvessels and of blood–brain barrier permeability, in a similar way to that found for several neurotransmitters and vasoactive mediators. Owing to the presence of tight junctions in the endothelial layer of brain cortex microvessels, it would even be of interest to ascertain whether, in this peculiar vascular region, the nociceptin/OP4 interaction occurs preferentially on the luminal or on the abluminal side of the blood–brain barrier.

In the present paper, we also showed that OP4 receptor activation by nociceptin induces phosphorylation of ERK isoforms, thus extending to the endothelial lineage the involvement of OP4 receptors in the activation of the MAP kinase cascade, which acts as a pathway that links plasma membrane receptor systems to diverse cellular processes—ranging from regulation of neuronal survival to cell differentiation and gene expression (Gutkind, 1998). In Chinese hamster ovary cells, signalling through ORL1 receptors has been found to involve ERK phosphorylation as a trigger for subsequent modulation of transcriptional factor activities (Lou et al., 1998). In our endothelial cells, the phosphorylation of both ERK isoforms exhibited a transient pattern, which could be due to various mechanisms ranging from the extracellular inactivation of nociceptin to the action of some specific phosphatase(s) or to well-known physiological processes such as desensitisation, receptor internalisation or downregulation, but might also reflect a peculiar intracellular signalling pathway (Marshall, 1995).

Our data prompt some speculation about a possible role of OP4 receptors in a specific endothelium-mediated regulation of blood–brain barrier permeability in brain capillaries and/or of vascular tone of large and peripheral blood vessels. The presence of functionally active OP4 receptors on endothelial membranes and their involvement in activating the MAP kinase cascade could explain the crucial role this system has in mediating changes in the vasoactive physiological status of various parts of the body.

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