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Divergent effects of corticotropin releasing hormone on endothelial cell nitric oxide synthase are associated with different expression of CRH type 1 and 2 receptors

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1 Endothelium is a target for an array of factors involved in inflammation. Endothelial cells express receptors for CRH, a neuropeptide produced during inflammation. We report both the concentration-dependent inhibitory effect of CRH upon cytokine-stimulated nitrite release by H5V murine endothelioma cells, and its stimulatory one in HUVEC cells.

2 Western blot analysis showed that CRH inhibits cytokine-stimulated iNOS protein in H5V cells, and, instead, potentiated it in HUVEC cells.

3 H5V cells expressed both CRH receptors (CRH-R1 and R2) mRNAs, whereas HUVEC cells expressed the CRH-R2 mRNA solely.

4 CRH increased medium nitrites and iNOS protein expression in H5V cells pretreated with the selective CRH-R1 antagonist CP 154,526. However, the selective CRH-R2 antagonist anti-Svg-30 failed to produce similar effects. In fact, anti-Svg-30 inhibited CRH-induced increase of nitrite release and iNOS expression in HUVEC cells.

5 Our results confirm the activating role of CRH on endothelial cells, although it suggests its possible inhibitory role in the late phase of the inflammatory response. NO-mediated effects of CRH on endothelial cells could be exploited in therapeutic strategies related to inflammatory and/or degenerative diseases.

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Abbreviations: anti-Svg-30, anti sauvagine 30; CRH, corticotropin releasing hormone; CRH-R1 and R2, corticotropin releasing hormone receptor 1 and receptor 2; H5V, murine endothelioma cell; HUVECs, human umbilical vein endothelial cells; iNOS, inducible nitric oxide synthase, NO, nitric oxide

Introduction

The 41-aminoacid hypothalamic peptide corticotropin releasing hormone (CRH) (Sawchenko & Swanson, 1985), a major activator of the hypothalamic-pituitary-adrenal (HPA) response to stress (Gold *et al*., 1986), has been detected in various tissues, including the adrenal (Andreis *et al*., 1991), the spleen (Webster & Desousa, 1983), the testis (Dufau *et al*., 1993), and the placenta (Petraglia *et al*., 1987). Interestingly, CRH has also been found in inflammatory cells from human blood (Stephanou *et al*., 1990).

CRH exerts pleiotropic effects, mediated by two different, adenylate cyclase-coupled, receptors, the CRH receptor types 1 and 2 (CRH-R1 and CRH-R2) (Chang *et al*., 1993; Chen *et al*., 1993; Perrin *et al*., 1993; 1995). CRH-R1 and CRH-R2 have been shown to produce different effects in the same murine behavioural model (Radulovic *et al*., 1999).

There are many data in support of CRH-R1-mediated proinflammatory effects of CRH (Webster *et al*., 1990; Singh & Fudenberg, 1988). To corroborate these data, many evidence shows that CRH is produced locally by fibroblasts and endothelial cells of the inflamed tissues (Karalis *et al*., 1991). In addition, CRH has been shown to activate human endothelial cells from the umbilical vein, an effect mediated by CRH-R2 receptors (Simoncini *et al*., 1999).

However, CRH has also been shown to inhibit pro-inflammatory prostaglandin production by endothelial cells from bovine aorta rings and from human foreskin fibroblasts (Fleisher-Berkovich & Danon, 1995), rather suggesting its possible anti-inflammatory role.

Vasodilator and cytotoxic phenomena occurring during inflammation are mediated, among others, by the gaseous mediator nitric oxide (NO) (Thiemmermann, 1994). Inflammatory NO synthesis depends upon the enzyme inducible NO synthase (iNOS) (Xin *et al*., 1992), an isoform of the constitutively expressed enzyme cNOS (Lamas *et al*., 1992).

iNOS is expressed as a result of the concerted antigen and/or cytokine-dependent activation of different cell types (Rao, 2000), including endothelial cells (Rao, 2000; Busse & Mulsch, 1990). It is of interest that autocrinally NO-activated endothelial cells are observed not only during the inflammatory response (Introna *et al*., 1994), but also during

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angiogenesis (Ziche *et al*., 1994), as well as in endothelial tumor cell proliferation (Ghigo *et al*., 1995).

In light of such biological relevance of iNOS and with the aim to define whether there is a link between CRH and iNOS and through which of the two receptor subtypes this occurs, we investigated the effects of CRH on cytokine-induced nitrite production and iNOS protein in the murine endothelioma H5V cells, a polyoma virus middle 'T' antigen transformed cell line, which expresses substantial amounts of iNOS (Ghigo *et al*., 1995; Bussolino *et al*., 1991). To assess possible differences in responsiveness to CRH, we also performed comparative experiments with HUVEC cells, known to express solely the CRH-R2 receptor (Simoncini *et al*., 1999).

Secondly, in order to further characterize the effects of CRH in both cell lines, we studied (1) the expression of CRH-R1 and -R2; and (2) the effects of the selective CRH-R1 antagonist CP 154,526 (Schulz *et al*., 1996), as well as the selective CRH-R2 antagonist anti-Svg-30 (Ruhmann *et al*., 1998) upon CRH-mediated nitrite release.

Methods

Tissue culture reagents, and chemicals

All the tissue culture supplies were purchased from Gibco (Life Technologies Italia srl, S. Giuliano Milanese, Milano, Italy), unless otherwise specified.

All chemicals were purchased from Sigma-Aldrich S.r.l. (Milano, Italy), unless otherwise specified. Human/rat corticotropin releasing hormone was obtained from Peninsula Laboratories Europe, LTD. (St. Helens, Merseyside, U.K.). Tumour necrosis factor (TNF)-α and interleukin (IL)-1ß were from Genzyme (Milano, Italy). Cytokine combination means a solution containing both TNF-α and IL-1β.

The polyclonal anti-mouse/human iNOS antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). M-MLV-reverse transcriptase, Taq polymerase and the primers for human and mouse CRH-R1, CRH-R2, iNOS and GADPH were purchased from Gibco, Life Technologies Italia srl, S. Giuliano Milanese, Milano, Italy.

The CRH-R1 antagonist CP 154,526, butyl-[2,5-dimethyl-7-(2,4,6 trimethylphenyl)-7H-pyrrolo [2,3-d] pyrimidin-4-yl]-ethyl-amine and the CRH-R2 antagonists anti-Sauvagine-30 ([DPhe^{11},His^{12}]Svg$_{(11-40)}$; anti-Svg-30,) were generous gifts respectively from Dr David W. Schulz (Pfizer Central Research; Groton, CT, U.S.A.), and Dr Jelena Radulovic (Department of Molecular Endocrinology; the Max Planck Institute for Experimental Medicine, Goettingen, Germany).

Cell cultures

The H5V murine endothelioma polyoma virus-middle 'T' antigen transformed, geneticine G-418 resistant cell line was a kind gift of Professor Alberto Mantovani, Istituto di Ricerche Farmacologiche Mario Negri, Milano; and University of Brescia, Italy). Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FCS, 200 u ml^{-1} penicillin, 50 μg ml^{-1} streptomycin mixture, 20 μM bacitracin, at 37°C, in 5% CO_2. The geneticin G-418 at a concentration of 800 μg ml^{-1} was periodically added to the cultures in order to maintain the lineage.

The human umbilical vein endothelial cells (HUVEC) were obtained from BioWhittaker Italia s.r.l. (Caravaggio, BG, Italy). Cells were grown in Endothelial Cell Basal Medium (EGM Bulletkit, BioWhittaker, Italia s.r.l., Caravaggio, BG, Italy) supplemented with 10 μg ml^{-1} human recombinant epidermal growth factor (hEGF), 1 μg ml^{-1} hydrocortisone, 50 μg ml^{-1} gentamicin, 50 ng ml^{-1} amphotericin-B, 20 μM bacitracin, 6 mg bovine brain extract (BBE), and 2% foetal bovine serum (FBS).

For nitrite assay, cells were plated into 24-well plastic plates in a volume of 1 ml DMEM well^{-1}, at a density of 2×10^5 cells well^{-1}. After 12 h, preincubation medium was replaced with the same, phenol-red free DMEM. Cells were then incubated with CRH (concentration range: 100 pM–1 μM), TNF-α (20 ng ml^{-1}), and IL-1ß (200 u ml^{-1}) or their combination, at different times. To assess cell viability, at the end of experiments cells were counted in a Hausser chamber after addition of trypan blue. Typically, viability was $\geqslant$99%).

All samples were run in triplicate; each experiment was repeated at least twice. At the end of the incubation, media were collected from each single well, rapidly frozen and stored at −80°C for nitrite measurement.

Medium nitrite assay (Green et al., 1982)

Sulfanilamide (1% in 3M HCl; w v^{-1}) and an equal volume of N-(1-naphthyl) ethylendiammine dihydrochloride (0.02% in water) were added to each sample (including a standard curve). Optical density (OD) was measured by means of a microplate reader ($\lambda=545$ nm) after 30 min incubation at room temperature. Concentration of nitrites in the medium was calculated according to the following formula: $OD_{545}\times$ linear regression of the standard curve. Inter- and intra-assay coefficient of variation were respectively 5% and 9.5%.

RT–PCR analysis for expression of murine and human CRH-R1 and CRH-R2 mRNAs

Total RNA was isolated by the guanidinium thiocyanate/chloroform extraction procedure (Chomczynski & Sacchi, 1987) either from cells incubated with cytokines and grown to confluence in 10 cm plastic Petri dishes, or from human placenta membranes (Karteris *et al*., 1998) obtained from women at term undergoing normal vaginal deliveries. The placenta were dissected off and immediately frozen in liquid nitrogen. Briefly, cells were lysed or tissue was thoroughly minced and homogenized with 1 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Sequentially, 0.1 ml of 2 M sodium acetate, pH 4, 1 ml of phenol, and 0.2 ml of chloroform-isoamyl alcohol mixture (49 : 1) were added to samples. The suspension was shaken vigorously for 10 s and cooled on ice for 10 min. Samples were centrifuged at $10{,}000\times g$ for 20 min. The resulting aqueous phase was transferred to a fresh tube and RNA precipitated with 1 ml of isopropanol at −20°C for at least 1 h. Centrifugation at $10{,}000\times g$ for 20 min was again performed and the resulting pellet of RNA was dissolved in 0.3 ml of solution D and precipitated with 1 ml of isopropanol at −20°C for 1 h.

After centrifugation for 10 min at 4°C the RNA pellet was washed in 75% of ethanol and then dissolved in 25 μl of distilled water. For first strand cDNA synthesis, 1 μg of total RNA was reverse-transcribed using 25 μg ml^{-1} oligo $(dT)_{12-18}$ primer in a final volume of 20 μl, in the presence of 200 units of M-MLV reverse transcriptase (Gibco). The reaction was carried out at 37°C for 1 h and heated at 95°C for 10 min, and subsequently for 5 min at 4°C. PCR was performed in a total volume of 20 μl, containing 2 μl of the cDNA, 5 pmol of each upstream and downstream primer, and 1.2 units of Taq polymerase (Gibco). The cycle program for: (a) mouse CRH-R1 primers consisted of 35 runs of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min.; (b) human CRH-R1 primers consisted of 35 runs of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 30 s; (c) mouse and human CRH-R2 primers consisted of 40 runs of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min; and (d) mouse and human GADPH primers consisted of 25 runs of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min. The cycle programme was preceded by an initial denaturation at 94°C for 3 min and followed by a final extension at 72°C for 10 min. PCR products were analysed by 1.0% agarose gel electrophoresis and visualized with ethidium bromide. The following RNA transcripts were detected *via* amplification of the corresponding cDNAs: (a) the mouse CRH-R1 using a primer pair composed of the sense primer 5′-GCCCTGCCCTGCC-TTTTTCTA-3′ and the antisense primer 5′-GTCATTAG-GATCCTGACGATG-3′ with an expected amplicon length of 744 base pairs; (b) the human CRH-R1 using a primer pair composed of the sense primer 5′-GCCCTGCC-CTGCCTTTTTCTA-3′ and the antisense primer 5′-GCTCATGGTTAGCTGGACCA-3′ with an expected amplicon length of 333 base pairs (c) the mouse and human CRH-R2 using a primer pair composed of the sense primer 5′-TGCTCAACTACCTGGGCCAC-3′ and the antisense primer 5′-GTCATTAGGATCCTGACGATG-3′ with an expected amplicon length of 522 base pairs; (d) mouse glyceraldehyde-3-phosphate dehydrogenase (GADPH) using the primer set composed of the sense 5′-GCCGCCTG-GTCACCAGGGCTG-3′ and antisense 5′-ATGGACTGTG-GTCATGAGCCC-3′, yielding an amplicon of 493-base pairs; (e) human glyceraldehyde-3-phosphate dehydrogenase (GADPH) using the primer set composed of the sense 5′-CCACCCATGGCAAATTCCATG-3′ and antisense 5′-TCTAGACGGCAGGTCAGGTCCACC-3′, yielding an amplicon of 598 base pairs.

Western blot analysis of iNOS

H5V cells and HUVECs treated with the cytokine combination, or with the addition of CRH, were grown at confluence in 60 mm plastic Petri dishes; cells were then lysed in NP-40 lysis buffer (HEPES 50 mM, pH 7.6, NaCl 150 mM, NaF 50 μM, EDTA 2 mM, sodium vanadate 1 mM, 1% NP-40, phenylmethylsulphonyl fluoride 2 mM). Cell debris was removed by centrifugation at $8000 \times g$ for 5 min, and the protein concentration was determined by the Bradford assay (Bradford, 1976). Cellular extracts (80 μg) were boiled for 10 min in SDS loading buffer (20% glycerol, 10% 2-mercaptoethanol, 4% SDS, 100 mM Tris-HCl pH 6.8, 0.2% bromophenol blue), separated by SDS–PAGE (8%), transferred to a nitrocellulose membrane, and probed with Ab anti-mouse/human iNOS (1 : 500) and with the secondary peroxidase-conjugated anti-rabbit Ab (1 : 1000) which was finally detected by enhanced chemiluminescence (ECL; Amersham Italia S.r.l., Milan, Italy).

Statistical analysis of results

Analysis of results was performed using one-way analysis of variance (ANOVA), followed by a Fisher's least significance test. A two-way ANOVA was applied when appropriate. Significance was accepted for a P value <0.05.

Results

Effects of corticotropin releasing hormone on cytokine-stimulated nitrite production from H5V and HUVEC cell cultures

We assessed the time-dependent effects of cytokines and CRH on nitrite release from H5V cells. Both IL-1ß and TNF-α induced increasing nitrite release from H5V when added to cell cultures.

Addition of 100 nM CRH significantly inhibited cytokine-mediated nitrite release. Maximal effects were observed after 24 h incubation. CRH alone failed to produce any change in production of nitrites from H5V cells (Figure 1). The effect of CRH on cytokine-stimulated nitrite release from H5V cells was concentration-dependent (data not shown), with maximal efficacy at 100 nM (IC_{50}: 1.2×100 nM) (Table 1).

Secondly, we assessed the effects of CRH upon cytokine-stimulated nitrite release from HUVEC. CRH (100 nM) did not interfere with cytokine-induced nitrite release from HUVEC cells.

However, CRH alone stimulated release of significant amounts of nitrites from HUVEC (Figure 2).

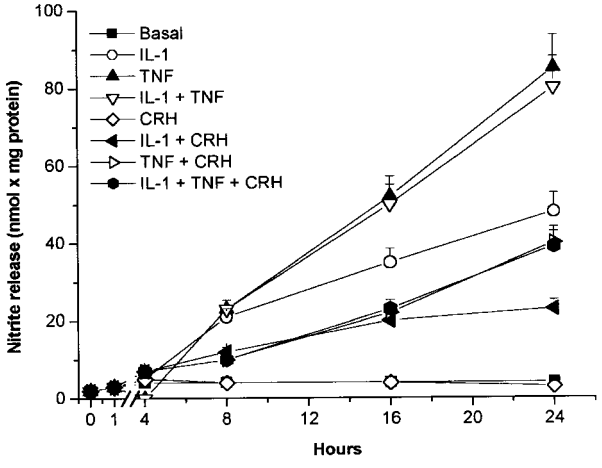

Figure 1 Time-related effects of IL-1β (200 u ml^{-1}), TNF-α (20 ng ml^{-1}), and CRH (100 nM), alone or in combination, upon nitrite release from H5V cell cultures. Vertical bars are means ±s.e.mean. *$P<0.05$ (two-way analysis of variance) vs basal values.

Table 1 Effects of TNF-α, IL-1β, and CRH upon nitrite release from endothelial cells

Treatment 24 h	Nitrite release (nmol × mg protein) H5V cells	HUVECs
Basal	4.0±0.4	2.0±0.2
IL-1β (200 u ml^{-1})	48±5.0	19±1.0
TNF-α (20 ng ml^{-1})	85±9.0	18±1.0
IL-1β+TNF-α	80±9.0	21±2.0
CRH (100 nM)	3.0±0.3	10±1.0**
CRH+IL-β	23±4.0*	21±2.5
CRH+TNF-α	40±5.0*	19±3.0
CRH+IL-1β+TNF-α	39±4.5*	23±3.5

*$P<0.05$ vs cytokines alone; **$P<0.05$ CRH vs basal (ANOVA).

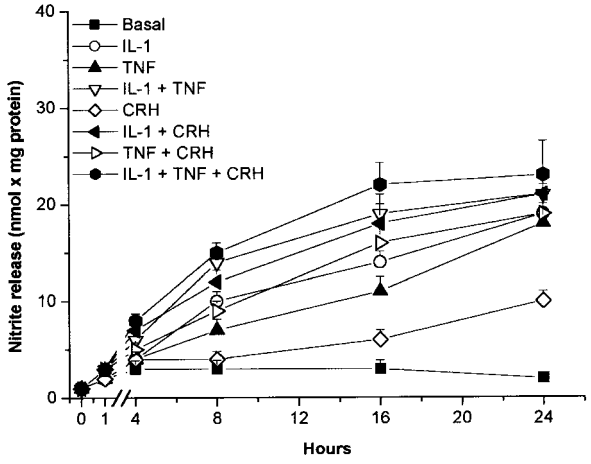

Figure 2 Time-related effects of IL-1β (200 u ml^{-1}), TNF-α (20 ng ml^{-1}), and CRH (100 nM), alone or in combination, upon nitrite release from HUVEC cultures. Vertical bars are means± s.e.mean. *$P<0.05$ (two-way analysis of variance) vs basal values.

Graded concentrations (range: 100 pM – 1 μM) of CRH did not induce any significantly additive effect on cytokine-induced nitrite release from HUVECs (EC_{50}: 7.2×10 nM) (data not shown), except for the concentration of 100 nM (and higher), which induced significant difference in nitrite release (Table 1).

When comparing the effects of cytokines and CRH at 24 h, it appeared that nitrite release induced by cytokines was significantly higher than basal values in both H5V and HUVEC cell cultures.

Nitrite production in response to 24 h incubation with cytokines was about 4 fold higher in H5V cells compared to HUVEC (Table 1).

Western blot analysis of iNOS protein in H5V and HUVEC cells

To detect changes in the iNOS protein levels induced by IL-1ß and TNF-α or combination of both in H5V cells, Western blot analysis was performed after 0, 1, 4, 8, 16 and 24 h treatment in the presence or absence of CRH (Figure 3A – C, upper parts).

A major band at 130 kDa, corresponding to the iNOS protein, was detected using an anti-mouse/human iNOS antibody. iNOS protein was not detected in untreated H5V cells (basal conditions, time 0). iNOS protein was detected after 4 h of treatment. Maximum level was observed after

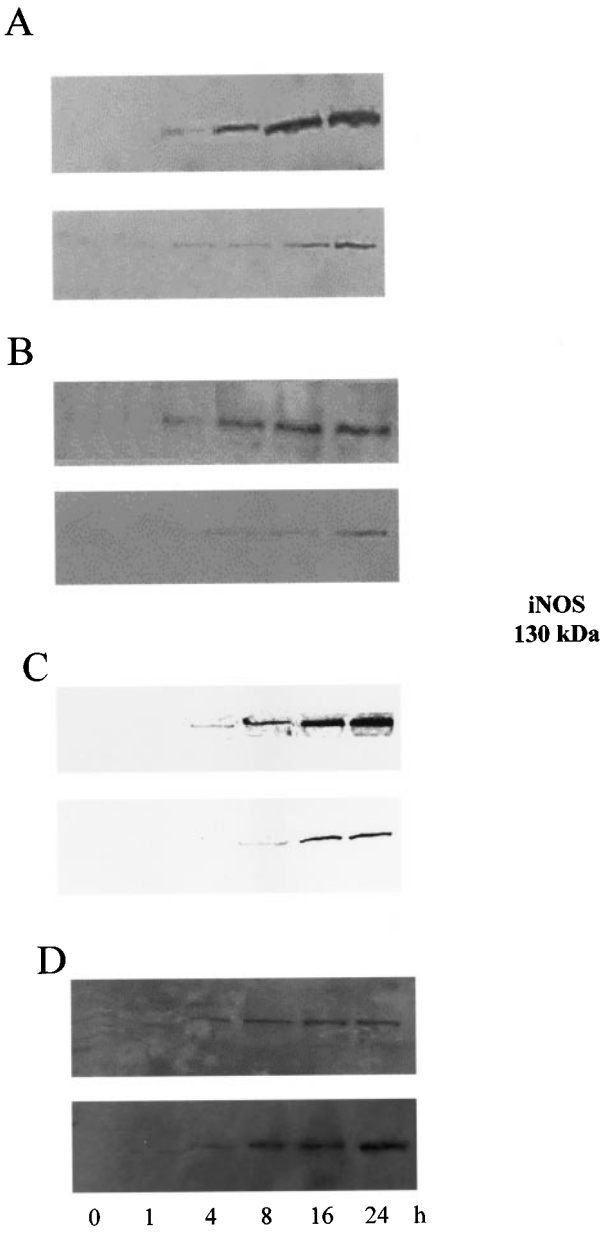

Figure 3 (A) Western blot analysis of iNOS protein expression at different times after incubation of H5V cells with TNF-α, alone (20 ng ml^{-1}; upper blot), or in the presence of CRH (100 nM; lower blot). (B) Western blot analysis of iNOS protein expression at different times after incubation of H5V cells with IL-1β, alone (200 u ml^{-1}; upper blot), or in the presence of CRH (100 nM; lower blot). (C) Western blot analysis of iNOS protein expression at different times after incubation of H5V cells with the combination TNF-α (20 ng ml^{-1})-IL-1β (200 u ml^{-1}) in the absence (upper blot), or in the presence of CRH (100 nM; lower blot). (D) Western blot analysis of iNOS protein expression at different times after incubation of HUVEC cells with the combination TNF-α (20 ng ml^{-1})–IL-1β (200 u ml^{-1}) (upper blot), and in the presence of CRH (100 nM; lower blot).

16–24 h in H5V cells. However, iNOS protein expression was markedly reduced in the presence of CRH (100 nM) in cytokine-stimulated H5V cells (Figure 3A–C, lower parts).

A similar pattern of iNOS protein expression was observed in HUVEC cells incubated with the combination IL-1ß/TNF-α (Figure 3D, upper part). CRH increased expression of iNOS at all the time points. (Figure 3D, lower part). In addition, CRH alone was able to induce weak expression of iNOS protein in unstimulated HUVEC cells (Figure 7).

RT–PCR analysis of CRH-R1 and CRH-R2 receptors mRNA in H5V and HUVEC cells

RT–PCR amplification analysis was performed to detect expression of both murine and human type 1 and 2 CRH receptor mRNAs.

RT–PCR amplification revealed the presence of both murine CRH-R1 and CRH-R2 mRNAs in untreated H5V cells, as well as in H5V cells treated 24 h with either IL-1β, TNF-α or CRH, or with a combination of the three substances (Figure 4A, B).

RT–PCR performed on total RNA extracted from cultured HUVEC and in the same experimental conditions as for H5V, revealed the presence of human CRH-R2 mRNA in cells treated with either IL-1β, TNF-α and CRH, or with the combination of the three substances (Figure 5B). Human CRH-R1 mRNA was undetectable in HUVEC. Positive control was represented by human placental mRNA (Figure 5A). As a negative control for the reaction, distilled water was used instead of cDNA.

Amplification of GADPH was used to justify the comparability of results in the murine and human CRH-R1 and R2 amplification analysis.

Effects of selective CRH-R1 and CRH-R2 receptor antagonists on nitrite release and iNOS protein expression in H5V and HUVEC cells

We have used the selective CRH-R1 and -R2 antagonists respectively CP 154,526 and anti-Svg-30 to characterize pharmacologically the effects of CRH. For this set of experiments, we used the time of 24 h, which corresponded to maximum expression of iNOS protein and nitrite release. In addition, we have used the concentration of CRH antagonists which completely inhibited CRH effects (concentration range tested: 1–1000 nM; data not shown).

The CRH-R1 antagonist CP 154,526 (1 μM) reverted the inhibitory effect of CRH upon TNF-α-stimulated nitrite release from H5V cells. The CRH-R2 antagonist anti-Svg-30 (1 μM) did not affect CRH-mediated nitrite increase inhibition in H5V cells.

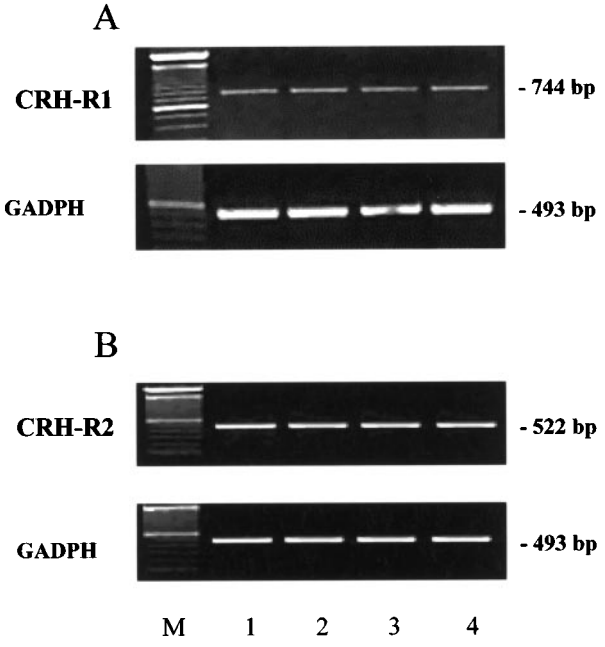

Figure 4 (A) RT–PCR analysis of murine CRH-R1 mRNA in H5V cells. M: Markers; Lane 1: Control (untreated cells); Lane 2: H5V cells treated for 24 h with 100 nM CRH; lane 3: H5V cells treated with the combination TNF-α (20 ng ml^{-1})–IL-1β (200 u ml^{-1}); Lane 4: H5V cells treated with the combination TNF-α (20 ng ml^{-1})–IL-1β (200 u ml^{-1})–CRH (100 nM). Lower gel: mouse GADPH control. (B) RT–PCR analysis of murine CRH-R2 mRNA in H5V cells. M: Markers; Lane 1: Control (untreated cells); Lane 2: H5V cells treated for 24 h with 100 nM CRH; lane 3: H5V cells treated with the combination TNF-α (20 ng ml^{-1})–IL-1β (200 u ml^{-1}); Lane 4: H5V cells treated with the combination TNF-α (20 ng ml^{-1})–IL-1β (200 u ml^{-1})–CRH (100 nM). Lower gel: mouse GADPH control.

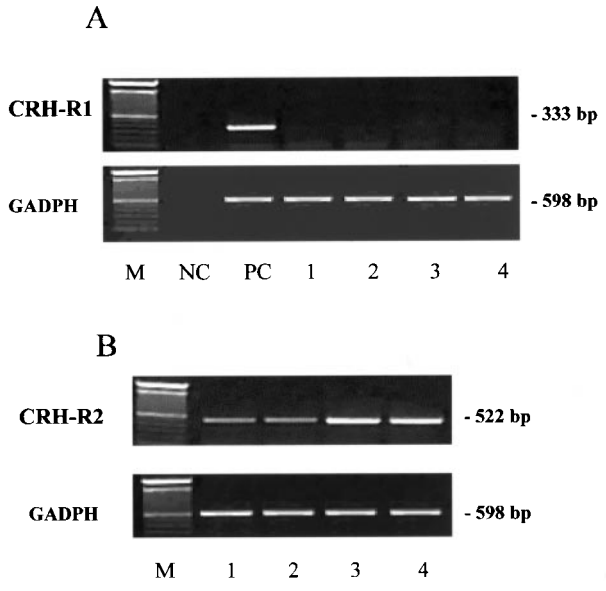

Figure 5 (A) RT–PCR analysis of human CRH-R1 mRNA in HUVEC cells. M: Markers; Lane NC negative control; Lane 2: Positive control (human placenta); Lane 3: HUVEC untreated; Lane 4: HUVEC cells treated for 24 h with 100 nM CRH; lane 5: HUVEC cells treated with the combination TNF-α (20 ng ml^{-1})–IL-1β (200 u ml^{-1}); Lane 6: HUVEC cells treated with the combination TNF-α (20 ng ml^{-1})–IL-1β (200 u ml^{-1})–CRH (100 nM). Lower gel: human GADPH control. (B) RT–PCR analysis of human CRH-R2 mRNA in HUVEC cells. M: Markers; Lane 1: Control (untreated cells); Lane 2: HUVEC cells treated for 24 h with 100 nM CRH; lane 3: HUVEC cells treated with the combination TNF-α (20 ng ml^{-1})–IL-1β (200 u ml^{-1}); Lane 4: HUVEC cells treated with the combination TNF-α (20 ng ml^{-1})–IL-1β (200 u ml^{-1})–CRH (100 nM). Lower gel: human GADPH control.

In parallel, iNOS protein expression induced by TNF-α was detectable only when CRH was added to cultures together with CP 154,526, but not in the absence of the antagonist.

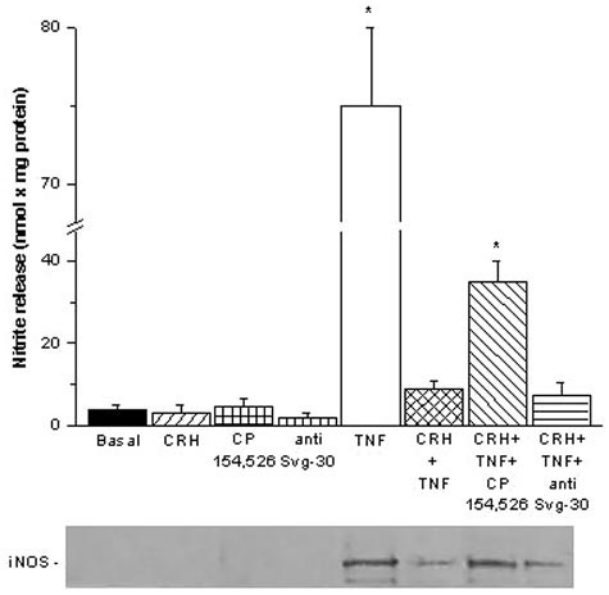

Figure 6 Effects of the CRH-R1 and CRH-R2 specific antagonists CP 154,526 (1 μM) and anti-Svg-30 (1 μM) upon nitrite release and iNOS protein expression (Western analysis) in H5V cell cultures. Incubation time was 24 h. Vertical bars are mean ± s.e.mean (one-way analysis of variance, followed by the Duncan's test); $*P<0.05$.

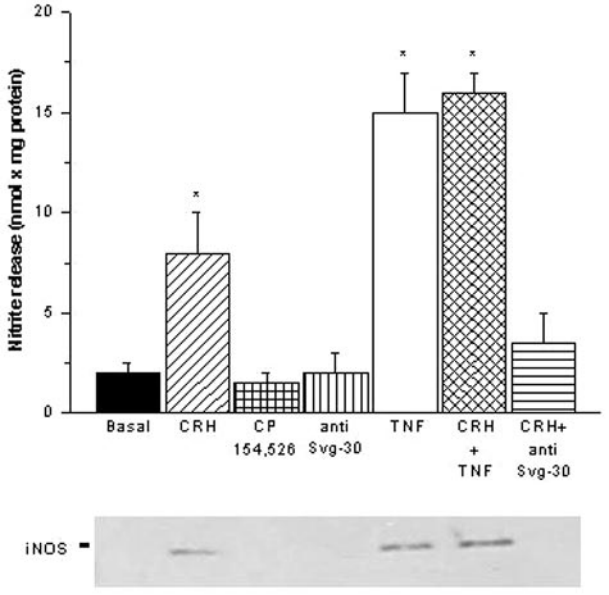

Figure 7 Effects of CRH-R1 and CRH-R2 specific antagonists CP 154,526 (1 μM) and anti-Svg-30 (1 μM) upon nitrite release and iNOS protein expression (Western analysis) in HUVEC cell cultures. Incubation time was 24 h. Vertical bars are mean ± s.e.mean (one-way analysis of variance, followed by the Duncan's test); $*P<0.05$.

Interestingly, the addition of the CRH-R2 antagonist anti-Svg-30 did not affect inhibition of iNOS expression induced by CRH in H5V cells (Figure 6).

In contrast, anti-Svg-30 inhibited both CRH-stimulated nitrite release and increased iNOS protein expression in HUVEC cells (Figure 7).

Discussion

Here, we demostrated that CRH displays divergent effects on cytokine-induced nitrite production and iNOS expression in different endothelial cell types.

In fact, we first showed that CRH inhibited cytokine-induced release of nitrites from the murine endothelioma cell line H5V.

Our results are in accordance with other data, showing that CRH improves experimental rabbit eyelid inflammation when injected locally (Mcloon & Wirtschafter, 1997), and decreases arachidonic acid metabolite production from cultured endothelial cells (Fleisher-Berkovich & Danon, 1995). In support of such endothelial NO-targeted inhibitory effects of CRH, it has also been reported that the peptide modulates endothelial NO-mediated placental vessel vasodilation (Clifton *et al*., 1995). In fact, CRH attenuated iNOS expression induced by IL-1β, TNF-α, or combination of the two cytokines in H5V cells.

Besides the indirect anti-inflammatory effect exerted by CRH *via* activation of the hypothalamic-pituitary-adrenal (HPA) axis (Munck *et al*., 1984), it is plausible that CRH produced locally in an inflammatory site (Karalis *et al*., 1991) could, on one side, act in an autocrine/paracrine manner to potentiate the HPA axis-mediated anti-inflammatory response.

On the other hand, we have shown that CRH enhanced nitrite release and induced iNOS protein expression in HUVEC cells.

The latter observation is corroborated by the reported proinflammatory effects of CRH described in the inflammation model of the rat carrageenin air pouch (Karalis *et al*., 1991), as well as by its presence in secretory granules of inflammatory cells (Stephanou *et al*., 1990). In addition, substantial amounts of CRH have been found in inflamed synovial tissues (Crofford *et al*., 1993), and CRH receptors, which are up-regulated in inflamed tissues (Mousa *et al*., 1996), have also been detected in macrophages (Audhya *et al*., 1991), whereas neutrophils respond to CRH with changes in their shape (Iavicoli *et al*., 1998).

Some authors have made an attempt to explain the discrepancy in the effects of CRH on inflammation by hypothesizing a biphasic effect of CRH, acting as an anti-inflammatory substance at low tissue concentrations, and eventually turning into proinflammatory when its concentration keeps increasing (Correa *et al*., 1997). Eventually, one can claim that such dual effect of CRH might depend upon down- or up-regulation of its receptors (Kapcala & De Souza, 1988; Sakai *et al*., 1997).

An additional rationale for our apparently divergent results could be found in the fact that H5V cells bear a transformed genotype (Ghigo *et al*., 1995), a feature that could be associated with loss of physiological responsiveness to stimulants.

Interestingly, RT–PCR revealed the presence of mRNAs for both CRH receptor types in H5V, whereas HUVEC cells expressed only CRH-R2 mRNA. In both cell types, expression of CRH receptors was independent of treatment with IL-1β or TNF-α.

It appears plausible that the different responsiveness of the two cell types to CRH might originate from such different expression of CRH receptors. In support of this hypothesis, Radulovic *et al*. reported in fact that the modulation of learning and anxiety in the mouse are regulated differentially by CRH-R1 and 2 (Radulovic *et al*., 1999).

Therefore, while CRH displays stimulatory effects upon normal resting endothelial cells, eventually potentiating the proinflammatory effects of IL-1β and TNF-α, it could reasonably, in contrast, inhibit the activity of cells permanently expressing the features of activated endothelial cells, such as H5V cells (Ghigo *et al*., 1995).

Further experiments were thus performed with specific CRH-R1 and -R2 receptors antagonists with the aim to better characterize such divergent effects of CRH on the two endothelial cell types and to eventually correlate them to such different CRH receptor pattern.

The selective CRH-R1 antagonist CP 154,526 restored nitrite release in parallel to iNOS expression in the presence of CRH in H5V cell cultures. We reasoned that the CRH-R1 blockade could results in exclusive binding of CRH to the other receptor, CRH-R2, thus restoring a response resembling to that observed in HUVEC cells (Simoncini *et al*., 1999).

As a counterproof, preincubation of H5V cells with the CRH-R2 receptor anti-Svg-30 resulted in unmodified nitrite release and iNOS expression in H5V cells, in contrast to HUVEC cells, where these parameters were inhibited by similar treatment.

Finally, CRH exerts differential effects on nitrite release and iNOS expression in endothelial cells. We have demonstrated that the effects of CRH are divergent in a way possibly related to the different CRH receptor pattern displayed by H5V and HUVEC cells. In fact, while H5V express both CRH-R1 and -R2 receptors, HUVECs express only the CRH-R2.

In conclusion, if on one side our data describing a iNOS-mediated endothelium-activating effect of CRH can be framed in the more general concept that CRH is a pro-inflammatory substance, they also suggest that the responsiveness of endothelial cells during an inflammatory response might vary depending upon their activation and/or differentiation state. In fact, endothelial cells may express different CRH receptors as a consequence of their activation, thus confering to the peptide anti-inflammatory effects.

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