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Research Article

Nitric oxide mediates histamine induced down-regulation of H_2 receptor mRNA and internalization of the receptor protein (R1)

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Abstract. During agonist-dependent long-term stimulation of cells, histamine receptor subtypes are frequently down-regulated. However, the mechanisms underlying the modulation of receptor expression during long-term histamine stimulation have yet to be resolved. Based on our recently reported results showing an H_1-mediated down-regulation of histamine H_2 receptor mRNA in endothelial cells, our aim was to characterize the mechanism controlling rapid and long-term histamine-mediated modulation of H_2 receptor expression in more detail. We were able to show that the histamine-induced down-regulation of H_2 receptor mRNA and cell surface expression lasting for 24 h was accompanied by augmentation of the receptor protein level in the cytoplasmatic fraction of endothelial cells for this time period. Furthermore, changes in receptor protein levels in whole-cell lysate were negligible, indicating that the rapid and prolonged modulation of cell surface H_2 receptor levels by histamine was regulated solely via internalization. The role of nitric oxide (NO) as a key mediator in histamine-stimulated cell responses was underlined by subsequent studies showing the attenuation of histamine-induced H_2 receptor mRNA down-regulation and protein trafficking following NO synthase isozyme inhibition.

Key words. Nitric oxide; histamine; G protein-coupled receptor; regulation; internalization.

Histamine, a biogenic amine, has been associated with a variety of early inflammatory responses such as expression of endothelial cell adhesion receptors, adhesion and rolling of leukocytes along endothelial cell walls, as well as migration of adherent leukocytes into the tissues at the site of inflammation. These effects have been shown to be mediated by the binding of histamine to specific G protein-coupled cell surface receptors (GPCRs), designated H_1 to H_4 [1–5]. Density, distribution and functional status of the specific histamine receptor subtypes are thereby important determinants for the receptiveness of cells and tissues to histamine stimulation. Binding and functional studies have provided a vast amount of indirect evidence for histamine receptor subtype expression in different tissues and organs. However, studies reporting direct detection or differential regulation of histamine receptor mRNA or protein expression are still scarce.

Differential modulation of histamine receptor expression has been observed particularly during inflammatory conditions, during cellular differentiation and following agonist stimulation. [6–9]. Our own investigations into the differential regulation of histamine receptor subtype expression demonstrated a rapid histamine induced down-regulation of H_2 receptor mRNA in endothelial cells that lasted for 24 h [10]. Although these studies underline the putative role of receptor expression and density for the re-

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sponsiveness of cells and tissues during pathophysiological conditions and agonist stimulation, they do not address the mechanism underlying the regulation of histamine receptor expression.

Regulation of GPCR expression has been shown to involve a variety of complex processes such as regulation of mRNA expression and protein synthesis, desensitization due to internalization and resensitization of receptor protein [11–13]. Desensitization is one of the processes that has been generally implicated as an important mechanism of short-term H_2 receptor regulation [14–17].

Our aim was to determine the mechanism of histamine-induced H_2 receptor regulation during long-term stimulation. To quantify the cell surface level of H_2 receptor protein and analyze the mechanisms underlying H_2 receptor regulation, we generated polyclonal anti-H_2 receptor antibodies and studied the cellular distribution of H_2 receptor protein by Western blot analysis and ELISA.

A key role of nitric oxide (NO) in histamine-induced inflammatory cell responses and gene expression in endothelial cells was reported by us previously [18, 19]. Furthermore, our results are in accordance with studies reporting that shock conditions such as vascular dilation [20], hyperemia [21, 22] and hypotension [23] are generated via H_1 [24, 25] or H_2 [21] receptor-stimulated formation of NO [26]. Additionally, a large number of studies have demonstrated a direct NO-induced regulation of gene expression [27–30].

The vast amount of evidence indicating NO as a key mediator in histamine-stimulated early inflammatory responses and the evidence showing NO-induced regulation of gene expression [27–29, 31–34] led us to analyze the role of NO in the rapid and long-term regulation of H_2 receptor mRNA and protein expression. The role of endogenous NO in histamine-induced regulation of H_2 receptor expression was established by using NO synthase (NOS) isozyme inhibitors during histamine stimulation of endothelial cells.

Our results indicate that the histamine-induced rapid and long-term down-regulation of H_2 receptor mRNA in endothelial cells is accompanied by a reduction in cell surface receptors. At the same time, we observed a significant increase in H_2 receptor protein in the cytoplasmic fraction. Furthermore, the significant down-regulation of H_2 receptor mRNA and cell surface receptor lasting for 24 h was not paralleled by a decrease in the overall amount of H_2 receptor protein, indicating that the down-regulation of cell surface receptor-induced levels during histamine stimulation was solely regulated via the internalization of the receptor.

Internalization as a long-term mechanism of cell surface reduction of GPCRs has to our knowledge not been reported to date.

Inhibition of NOS isozymes during histamine stimulation prevented the histamine-induced down-regulation of H_2 receptor mRNA and the internalization of H_2 receptor protein. Our previous results showing a key role of the histamine-stimulated activation of NOS isozymes and the synthesis of endogenous NO for histamine-induced cellular responses were thereby underlined. The precise mechanism by which NO mediates the regulation of H_2 receptor expression has yet to be elucidated.

Materials and methods

Cell culture and stimulation procedures

Human umbilical vein endothelial cells (HUVECs; Clonetics, Heidelberg, Germany) were cultured in EBM medium (Clonetics) supplemented with 0.01 µg/ml human epidermal growth factor (hEGF), 1 µg/ml hydrocortisone, 50 µg/ml gentamycin sulfate, 50 µg/ml amphotericine B, 12 µg/ml bovine brain extract and 10% fetal calf serum (FCS) (Life Technologies, Egenstein, Germany) at 37 °C, 5% CO_2, 95% air. Medium was exchanged every 48 h. Endothelial cell cultures were used at the second through the seventh passage.

For stimulation, culture medium was removed from tightly confluent cells and substituted by medium containing 1 mM histamine (Sigma, Deisenhofen, Germany). Histamine was dissolved in medium. The specificity of effects induced by histamine at this concentration was determined previously [10, 18, 19]. The histamine-induced effect was also shown to be concentration dependent [10]. NOS isozymes were inhibited by incubating cells with S-ethylisothiourea (10 nM, 0.1 µM, 1 µM, 10 µM) as well as L-NAME (5 nM, 50 nM, 0.5 µM) (Calbiochem, Bad Soden, Germany) during histamine stimulation. NOS isozyme inhibitors were added to endothelial cells 10 min prior to histamine. Histamine and NOS inhibitor concentrations have been determined previously [18, 19]. Cells were incubated for various time periods. Stimulation was terminated with PBS (NaCl 120 mM, KH_2PO_4 2.7 mM, Na_2HPO_4 10 mM).

Designing a standard RNA for competitive RT-PCR analysis

For competitive RT-PCR analysis, each reaction should contain a known amount of a competitor RNA that undergoes amplification with the same primers as does the endogenous mRNA and should yield products distinguishable from those derived from endogenous templates on gel electrophoresis. To construct vectors from which such competitor RNAs are generated, we purchased two single-stranded DNA sequences containing primer annealing sites for PCR amplification of the H_2 receptor gene. In addition, each single-stranded DNA sequence possessed 20 nucleotides which were complementary to the 5′ or 3′ end of a double-stranded nonsense DNA strand. The size of the double-stranded nonsense DNA

strand was 282 bp. By using the following H_2 receptor primer sequences: sense 5′ATGGCACCCAATGGCACAGC3′, antisense 5′ACTTGCAGGACAGCTGGTAG3′, PCR was performed, producing an amplification product 322 bp in length. The composition of the assay and the reaction conditions were as follows: 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH8.8, 1 mmol/l dNTPs, 1 U Taq polymerase + 0.02 U PWO polymerase/50 µl, 2 ng double-stranded nonsense DNA and 20 nmol/l of H_2 receptor sense and antisense primer. After a denaturation step at 95 °C for 5 min, 15 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min were performed with a finishing elongation step at 72 °C for 7 min. To exclude mismatches due to Taq polymerase amplification, the resulting PCR product (322 bp) was sequenced using the ABI Prism 277 Automated Sequencer (Seqlab, Göttingen, Germany). Subsequently, the PCR product was cloned into the plasmid pCR4-TOPO, (Invitrogen, Karlsruhe, Germany) containing a T3/T7 RNA promotor sequence. RNA transcripts of this internal standard sequence were prepared as run-off transcripts by linearization at the *NOT1* site (Roche, Mannheim, Germany). In vitro transcription was performed using a commercially available kit (T3 or T4 RNA polymerase; Roche, Mannheim, Germany) according to the manufacturer's instructions. The DNA template was digested with 10 U DNaseI (Promega, Madison, Wisc.) at 37 °C for 20 min. After ethanol precipitation, the amount of internal standard RNA was determined at 260 nm. Absence of contaminating traces of remaining DNA template interfering in the competitive reverse transcription PCR assay was verified by subjecting the RNA to PCR amplification omitting the reverse transcription reaction.

Competitive RT-PCR

RNA was extracted from endothelial cells using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instruction. Reverse transcription and PCR were performed using the Titan One Tube RT-PCR System (Roche). Total RNA was added to all components of the system in a total volume of 50 µl: 0.2 mM each of dATP, dCTP, dTTP and dGTP, 0.4 µM sense and antisense primer, 40 U RNase inhibitor (Eppendorf, Hamburg, Germany) in a buffer of 100 mM Tris-acetate (pH 6.5), 10 µl 5 × RT-PCR buffer (7.5 mM $MgCl_2$), 2.5 µl dithiolthreitol (DTT) solution (100 mM) and 1 µl Expand High Fidelity Enzyme consisting of Taq DNA Polymerase, Pwo DNA Polymerase and AMV Reverse Transcriptase. cDNA synthesis was carried out in three different 50-µl reactions each containing 0.1 µg of total RNA and various amounts of corresponding internal standard RNA (0.1, 1, 5 and 10 pg). The temperature cycles for amplifying H_2 receptor cDNA consisted of: 30 min reverse transcriptase reaction at 55 °C, 3 min of denaturing at 94 °C (1 cycle), 30 s of denaturing at 94 °C, 30 s primer annealing at 60 °C, 90 s of primer extension at 68 °C (35 cycles), 7 min primer extension at 68 °C (1 cycle) (Thermocycler PC-960; LTF-Labortechnik, Wasserburg, Germany). Amplification products were 252 bp in length for the target RNA and 322 bp for the internal standard RNA.

For semi-quantitative analysis, RT-PCR conditions were essentially the same. GAPDH was amplified as an internal control. The sequences of GAPDH primer sequences utilized were: sense 5′CCATGGAGAAGGCTGGGG3′, antisense 5′CAAAGTTGTCATGGATGACC3′.

For PCR analysis, 10-µl aliquots of the PCR product were fractionated by horizontal gel electrophoresis on a 1.5% agarose ethidium bromide-stained gel. PCR products were quantified using high-performance liquid chromatography (HPLC).

High-performance liquid chromatography

PCR reaction products were quantified by size fractionation through anionic exchange HPLC in a Waters LCM1 plus system (Waters Cooperation, Eschborn, Germany). This includes two W600 pumps, an automated gradient controller, a W715 autosampler, a W486 UV detector and a W746 data module for data analysis. The column was calibrated for 10–12 min with a buffer ratio 70/30 buffer A (25 mmol/l Tris-HCl, pH 7.8, 1 mol/l NaCl)/buffer B (25 mmol/l Tris-HCl, pH 7.8). The total flow rate was 1 ml/min. Ten microliters of the PCR reaction probe was loaded on a prepacked DEAE-bonded nonporous resin particle column (diameter: 2.5 µm). PCR products were separated by the following gradient program: 0 min, 70/30 buffer A/B; 0.5 min, 60/40 A/B; 6.5 min, 50/50 A/B; 6.51 min, 0/100 A/B; 7.9 min, 0/100 A/B; 8 min, 70/30 A/B; 15 min, 70/30 A/B. The elution profile was assessed at 260 nm. Peak areas corresponding to the PCR products, detected at 260 nm by HPLC, were integrated and normalized to levels of GAPDH message (Millenium Software, Waters Cooperation).

Quantification of H_2 receptor mRNA

The initial amount of target and standard RNA was quantified by regression analysis of the integrated peak areas corresponding to the two respective PCR products. The point of equivalence was determined by blotting the ratio of standard to target DNA versus the amount of standard RNA included in each reaction in a semi-logarithmic diagram. The point of equivalence represented the equimolar ratio of molecules of target and standard RNA initially present in the assay. The results were corrected for the different sizes of the PCR products.

Antibodies

Rabbit polyclonal antibodies against the H_2 receptor were raised using a keyhole limpet hemocyanin-conjugated synthetic peptide with the sequence CSRNETSKGN-

HTTSKC (PH2) (Seqlab, Goettingen, Germany) as immunogen. The peptide (25 µg) in PBS was emulsified with an equal volume of Freund's complete adjuvant (500 µl) and injected subcutaneously. Three subsequent injections given at 2-week intervals contained Freund's incomplete adjuvant. The rabbits were bled, serum-recovered, and IgG was purified by affinity chromatography on protein A-Sepharose (Sigma).

Immunocytochemistry

HUVEC monolayers were grown to confluence in Lab-Tek II chamber slides (Nunc, Naperville, Ill.) in supplemented EBM medium (Clonetics) as described above. Confluent cells were incubated in PBS supplemented with 2% bovine serum albumin (BSA), 2% non-fat dry milk (Sigma) and 0.03% NaN_3 (Sigma) for 1 h at room temperature. Subsequently, cells were incubated with rabbit polyclonal anti-H_2 receptor serum at a dilution of 1:2000 overnight at 4 °C in PBS. The following day, cells were incubated with polyclonal donkey anti-rabbit $F(ab')_2$ fragment-Cy3-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, Pa.). Between individual incubation steps, cells were washed with PBS. Finally, cells were mounted with a glass coverslip using Aquatex (Merck, Darmstadt, Germany). The labeled cells were subsequently viewed with a fluorescent microscope (Carl Zeiss, Jena, Germany) at 578–618 nm; images were captured using the Meta View imaging program (Universal Imaging Corp., Downingtown, Pa.).

ELISA

HUVECS were grown on ELISA plates to confluence. Plates were blocked with BSA (5%, w/v) at room temperature for 2 h. After three washes with PBS, serum raised to PH2 was diluted in BSA (1% w/v) and added to the plates (dilution 1:200/well) for 1 h at room temperature. The plates were then washed three times with PBS, and horseradish peroxidase-labeled goat anti-rabbit IgG was added (1:2000 dilution, 100 µl/well) for 1 h at room temperature. After further washes with PBS, 100 µl of a chromogenic substrate solution (580 µl/ml tetramethylbenzidine and 0.0001% H_2O_2 in 0.1 M sodium acetate buffer, pH 5.2) was added per well, and color development was allowed to proceed for 10 min in the dark. The reaction was stopped by the addition of 2 M H_2SO_4 (50 µl/well), and the absorption at 450 nm was measured using an ELISA plate reader MR 5000/MR 7000 (Dynateeck Laboratories, Denkendorf, Germany).

Preparation of plasma membranes and cytoplasmatic fractions

The method used to prepare crude plasma membranes allows the separation of plasma membranes and a cytoplasmatic fraction still containing cellular membranes such as endoplasmatic reticulum, mitochondria, lysosomes and Golgi [35]. Cytosolic lysis buffer (CLB) 2.5 ml; 10 mM HEPES, 10 mM NaCl, 1 mM KCl, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM PMSF and 100 U/mL aprotinin) containing EDTA (5 mM) was added to confluent cells in 25-cm^2 Falcon flasks (Becton Dickinson, Heidelberg, Germany) and incubated for 5 min. Cells were removed with a rubber policeman. Lysate from three Falcon flasks was transferred to a 15-ml Falcon tube (Becton Dickinson) and disrupted by sonication: 2 × 10 s at 70% power (Bandolin Sonoplus, HD 2070; Bandolin, Berlin, Germany). After removing detritus and nuclei by centrifugation at 50 g, plasma membranes were pelleted at 7500 g for 15 min at 4 °C. The supernatant was collected for further preparation of protein from the cytoplamatic fraction as described below. Membrane pellets were resuspended in 100–200 µl of a triple-detergent lysis buffer [RIPA buffer: 1% Igepal (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (Sigma), 1mM PMSF (Sigma) in PBs]. Protein content was measured using the DC Protein Assay (Bio Rad, Hercules, Calif.) according to the manufacturer's instructions. This procedure yielded 200 µg membrane protein/5 × 10^6 cells.

Protein from the cytoplasmatic fraction was precipitated by adding 1 ml chloroform (Sigma) und 4 ml methanol (Baker, Deventer, The Netherlands). After extensive vortexing, the solution was centrifuged at 3000 g for 5 min at 4 °C. Subsequently, protein was concentrated in the interphase. Supernatant was removed and 3 ml methanol was added. Protein was precipitated by centrifugation at 3000 g. The supernatant was removed and the protein pellet dried. Dry protein was resuspended in 1 ml RIPA buffer. A protein yield of ca 1 mg/75 cm^2 was determined.

Whole-cell lysate was obtained by lysing cells directly in RIPA buffer (660–900 µl). The surface of the Falcon flasks was scraped with a rubber policeman, the lysate removed to a microcentrifuge tube, homogenized through a 21-gauge needle and incubated for 60 min on ice. Subsequently, samples were centrifuged at 10,000 g for 10 min at 4 °C to remove debris. Supernatant was stored at –80 °C.

Western blot analysis

Quantitative analysis of H_2 receptor protein expression in different cellular fractions was performed by Western blot. Each lane of a 4–12% NuPAGE gel (Invitrogen, Karlsruhe, Germany) was loaded with 2.5–5 µg of protein and electrophoresed at 200 V. Protein transfer to nitrocellulose membranes (Hybond ECL; Amersham Bioscience Europe, Freiburg, Germany) was performed using semi-dry conditions. Subsequently, membranes were incubated with anti-H_2 receptor serum at a 1:200 dilution at 4 °C overnight and with a secondary antibody (goat anti-rabbit IgG1 horseradish peroxidase-conjugated antibody; DAKO, Carpinteria, Calif.) at a dilution of

1:10,000. The reaction was visualized on BIOMAX light-1 film (Kodak, Rochester, Minn.) after incubation of membranes with luminol-based chemiluminescence reagent for 1 min. To normalize experiments, membranes were stripped with 100 mM glycine at pH 2.5 for 10 min and reprobed with an antibody against actin (Santa Cruz Biotechnolog, Santa Cruz, Calif.). Results were scanned and quantified by Gel-Pro Analyser (Media Cybernetics, Silver Spring, Md.).

Data analysis

In general, results were normalized against internal controls and displayed as percentage of control values derived from untreated endothelial cells. Differences in time course between control values and the different time points were tested using the paired Student's t test. Due to multiple comparisons, only p values ≤ 0.02 were considered significant. The results are displayed as means (± SE).

Results

Characterization of H_2 receptor expression in untreated endothelial cells

Expression of H_2 receptor gene and protein in untreated endothelial cells was examined by competitive RT-PCR, Western blot analysis and immunofluorescent staining of cells. Equal amplification effiency was shown for competitive RT-PCR reactions of sample and standard RNA during co-amplification in single reaction tubes (data not shown). All amplification products proved to be in the exponential amplification phase at 37 cycles, as determined by HPLC. Omission of reverse transcriptase mix yielded no PCR signals. A representative gel of competitive RT-PCR products is shown in figure 1a. The amount of H_2 mRNA in HUVECs was determined by plotting the logarithmic ratio of HPLC-quantified PCR products (standard/endogenous mRNA) versus the logarithm of standard mRNA (fig. 1b). The amount of H_2 transcript in HUVECs was determined to be 196 ± 9 amol/µg total RNA.

Polyclonal antibodies raised against peptides corresponding to 16 amino acids of the second extracellular loop of H_2 receptors, designated PH2, were used to detect corresponding proteins by Western blotting and immunostaining. When blots were stained with anti-H_2 receptor antibodies, two distinct signals were observed in whole-cell lysate, as well as membrane fractions of HUVECs (fig. 2a). In the cytoplasmatic fraction, a single signal was detected (compare also fig. 4c). The smaller, more pronounced signal with a size of approximately 38 kDa coincides with the presumed size of the H_2 receptor (http://www.expasy.org/tools/peptide-mass.html). Preincubation of serum with PH2 caused the disappearance of all bands, indicating the specificity of the signals.

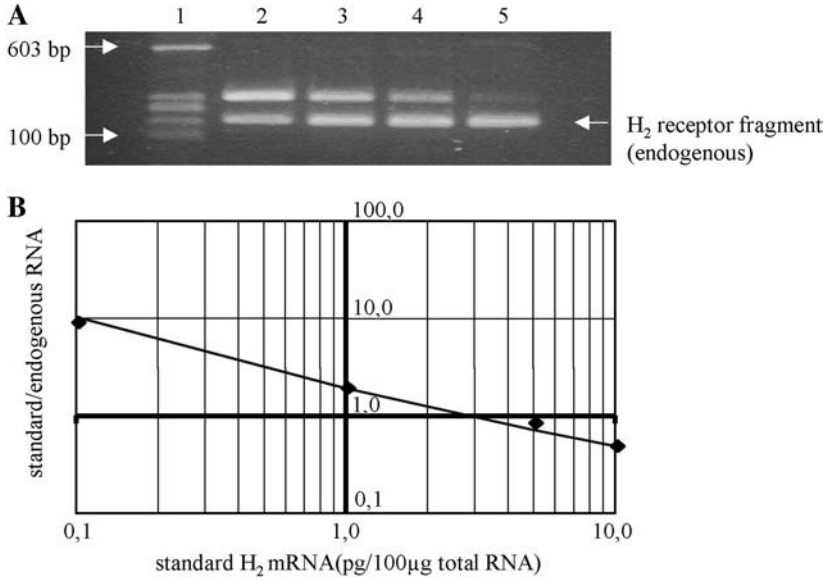

Figure 1. Quantitative competitive RT-PCR analysis of H_2 receptor mRNA levels expressed by HUVECs. Aliquots (0.1 µg) of total RNA isolated from HUVECs were reverse-transcribed and PCR-amplified with H_2 receptor-specific primers. (*A*) The products were separated on 1.5% agarose gels and visualized by ethidium bromide staining; lanes 2–5 represent co-amplification of sample RNA with 0.1, 1, 5 and 10 pg of H_2 receptor RNA mimic. Lane 1 represents bands corresponding to the DNA Molecular Weight Marker IX (Roche). The relative amounts corresponding to the target and standard PCR products were quantified using HPLC. (*B*) The ratio of standard to target cDNA was graphed as a function of the log of the amount of standard RNA included in each reaction; amplification product of endogenous H_2 receptor RNA, 252 bp; amplification product of added standard RNA 322 bp. The experiments were performed at least three times independently. A representative result is shown.

Reports showing the formation of a reducing SDS-PAGE-resistant formation of β_2AR homodimers [36] convey the feasible notion that the larger rather weak signal is a dimerization product. However, the formation of H_2 homodimers can be ruled out due to the size of the second band (approx. 60 kDa). Furthermore, taking into account that the larger signal was not observed in the cytoplasmatic fraction, we would like to suggest that the larger signal might be a result of heterodimer formation at the membrane level. However, whether the larger-size signal is the result of dimerization with a smaller protein or is due to posttranslational modifications of the membrane-bound H_2 receptor remains to be elucidated. No signals were observed when blots were incubated with preimmune serum.

Immunostaining of untreated HUVECs with histamine H_2 receptor antiserum allowed cellular localization of the histamine H_2 receptor. Signals were concentrated at the plasma membrane and the distribution was essentially homogenous (fig. 2b). All specific staining was abolished following preincubation of the antisera with the corresponding immunogenic PH2 peptides. Incubation of cells with the respective rabbit preimmune serum did not generate specific signals (results not shown).

Fixation of cells with paraformaldehyde or methanol prior to immunostaining forestalled the appearance of specific signals. This could indicate that the antibodies are susceptible to alterations of the corresponding epitopes, such as masking, which might be caused by the fixation procedure.

Time-dependent histamine-induced H_2 receptor regulation and internalization

In previous studies, we demonstrated a pronounced histamine-induced down-regulation of histamine H_2 receptor mRNA in HUVECs that was mediated via the H_1 receptor [10]. It was our aim to investigate the effect of histamine-induced H_2 receptor mRNA down-regulation on H_2 receptor protein levels in HUVECs. Therefore, the time course of histamine-induced modulation of cell surface

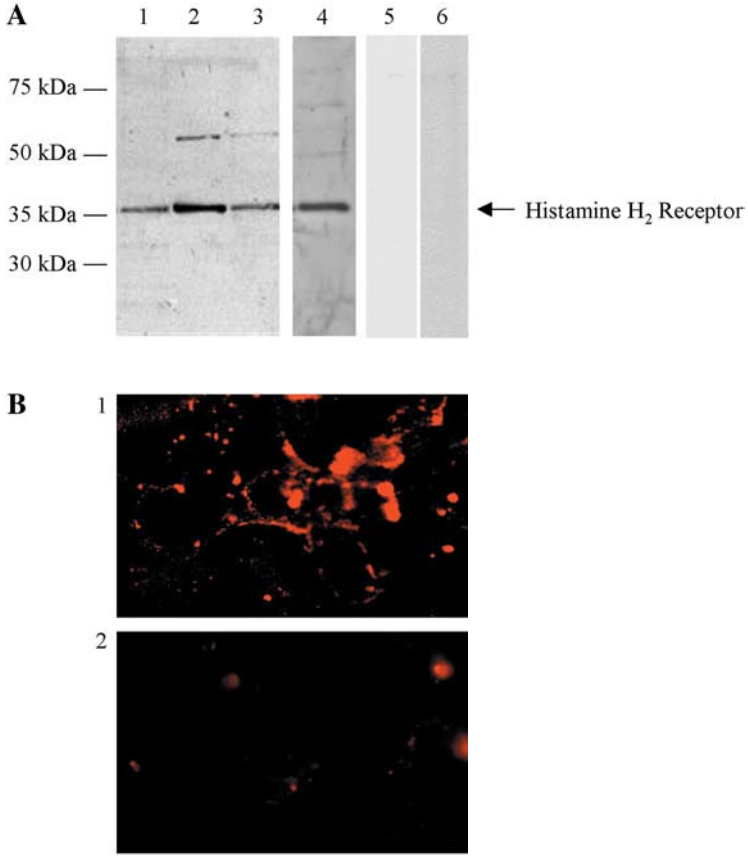

Figure 2. Immunoblotting and immunostaining of H_2 receptors expressed in HUVECs and CHOrH2. (*A*) Whole-cell lysates (1) of HUVECs were prepared and fractionated into membrane (2) and cytoplasmatic (3) fractions as described in Materials and methods. Whole-cell lysate of CHOrH2 cells was prepared as a control (4). Aliquots were subjected to SDS-PAGE electrophoresis. The presence of H_2 receptor protein in the samples was detected by probing respective membranes with polyclonal anti-H_2 receptor antibodies. Simultaneously, blots of whole-cell lysate were incubated with either preimmune serum (5) or serum preincubated with the peptide used for raising anti-H_2 receptor antibodies, designated PH2 (6): (*B*) Living cells were processed as described in Materials and methods and stained with serum (1) and serum preincubated with PH2 (2). Data are representative images of three independent experiments.

H_2 receptor density as determined by ELISA and Western blot analysis was compared to the time course of H_2 receptor mRNA down-regulation quantified by competitive RT-PCR analysis. The quantitative analysis of histamine-induced H_2 receptor mRNA down-regulation was in accordance with the results obtained in earlier studies. Within 2 h of histamine stimulation, H_2 receptor mRNA expression reached its lowest level at 43.9 ± 1.5% ($p \leq 0.001$) compared to the control level. After 8 h of stimulation, we observed a steady increase in H_2 receptor message. However, even after 48 h of histamine stimulation, the level of H_2 receptor message did not reach control values. Histamine-induced down-regulation of H_2 receptor mRNA was still statistically significant (48 h: 74.4 ± 3.2%; $p \leq 0.002$).

When we compared the time course of histamine-induced modulation of H_2 receptor protein by ELISA to the modulation of H_2 receptor mRNA, the decrease in mRNA level preceded the loss of H_2 receptor protein from the cell surface. Loss of H_2 receptor from the cell surface was comparatively slow, with the most pronounced reduction after 16 h of histamine stimulation (–48.3 ± 6.4%; $p \leq 0.001$). After 48 h of histamine stimulation, cell surface receptor expression increased to 81.5 ± 5.2% ($p \leq 0.02$; fig. 3b), but remained lower than H_2 receptor cell surface expression in unstimulated cells. The time course of cell surface H_2 receptor expression determined by ELISA was confirmed by Western blot analysis of membrane fractions (fig. 4a), indicating the newly generated H_2 receptor antibodies to be beneficial for the

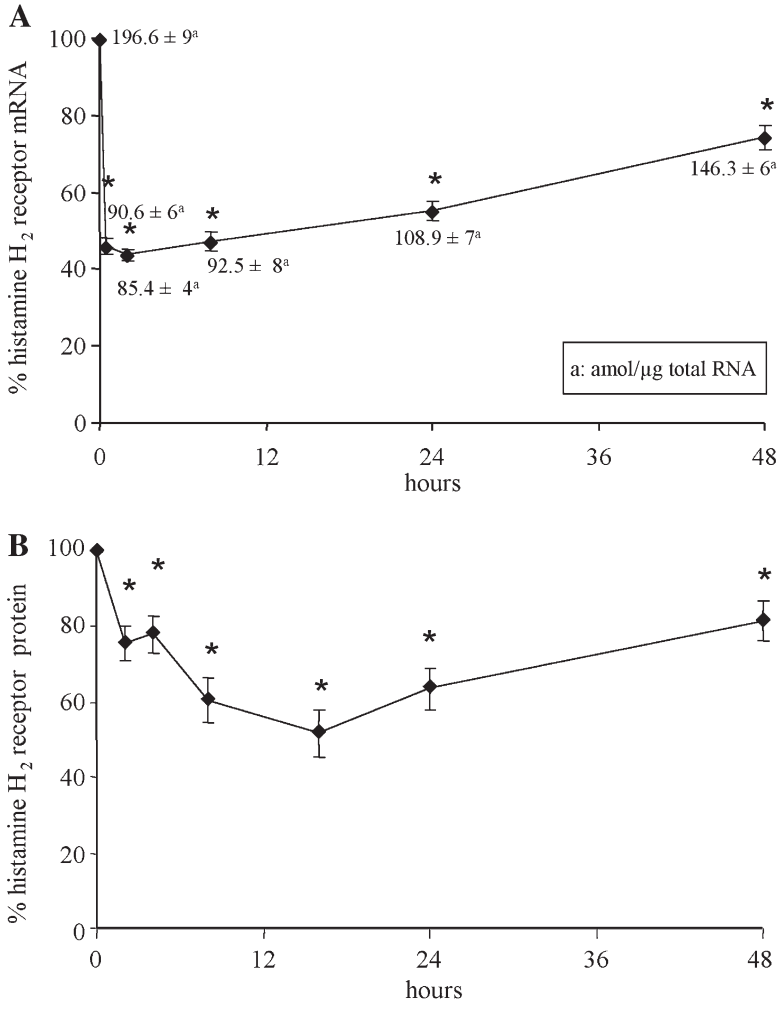

Figure 3. Time-dependent effect of histamine on H_2 receptor expression. (*A*) HUVECs were stimulated with histamine (1 mM) for 2–48 h. Aliquots (0.1 µg) of total RNA and increasing concentrations of standard mRNA were reverse-transcribed and PCR-amplified for 35 cycles with H_2 receptor-specific primers (competitive RT-PCR). The relative amounts corresponding to the target and standard PCR products were quantified using HPLC. Results in the graphs are shown as percentage induction of controls. Specific concentrations of endogenous H_2 receptor mRNA are indicated at the specific time points. (*B*) Cell surface H_2 receptor expression was determined by ELISA. Living cells grown to confluence were incubated with anti-H_2 receptor antibodies and quantified with an ELISA reader. (*A*, *B*) Data of a minimum of six experiments were averaged and are represented as the mean ± SE, expressed as percentage decrease with respect to unstimulated HUVECs. * $p < 0.02$ versus time 0.

quantification of H_2 receptor protein by ELISA and Western blot analysis.

Further analysis of the cellular localization of the H_2 receptor by Western blot analysis revealed that the reduction in cell surface receptor expression is accompanied by a progressive augmentation of H_2 receptor protein in the cytoplasmic fractions during the first 8 h of histamine stimulation (fig. 4b), indicating the long-term down-regulation of H_2 receptor cell surface expression to be due to the internalization of the protein. Furthermore, the enhanced level of H_2 receptor signal in the membrane fraction after 24 h of histamine induction is accompanied by a loss of receptor protein in the cytoplasmic fraction. The significant increase in H_2 receptor level in the cytoplasmic fraction after 48 h of histamine stimulation might be linked to augmentation of H_2 receptor transcript at this time point (compare fig. 3a).

The overall H_2 receptor protein level in whole-cell lysate remained constant during the first 4 h of histamine incubation (fig. 4c). However, after this time point, a slight change in the overall cellular H_2 receptor protein level was observed. Although this increase seems to be concomitant with the slow increase in H_2 receptor transcript after 8 h of histamine stimulation, it was not statistically

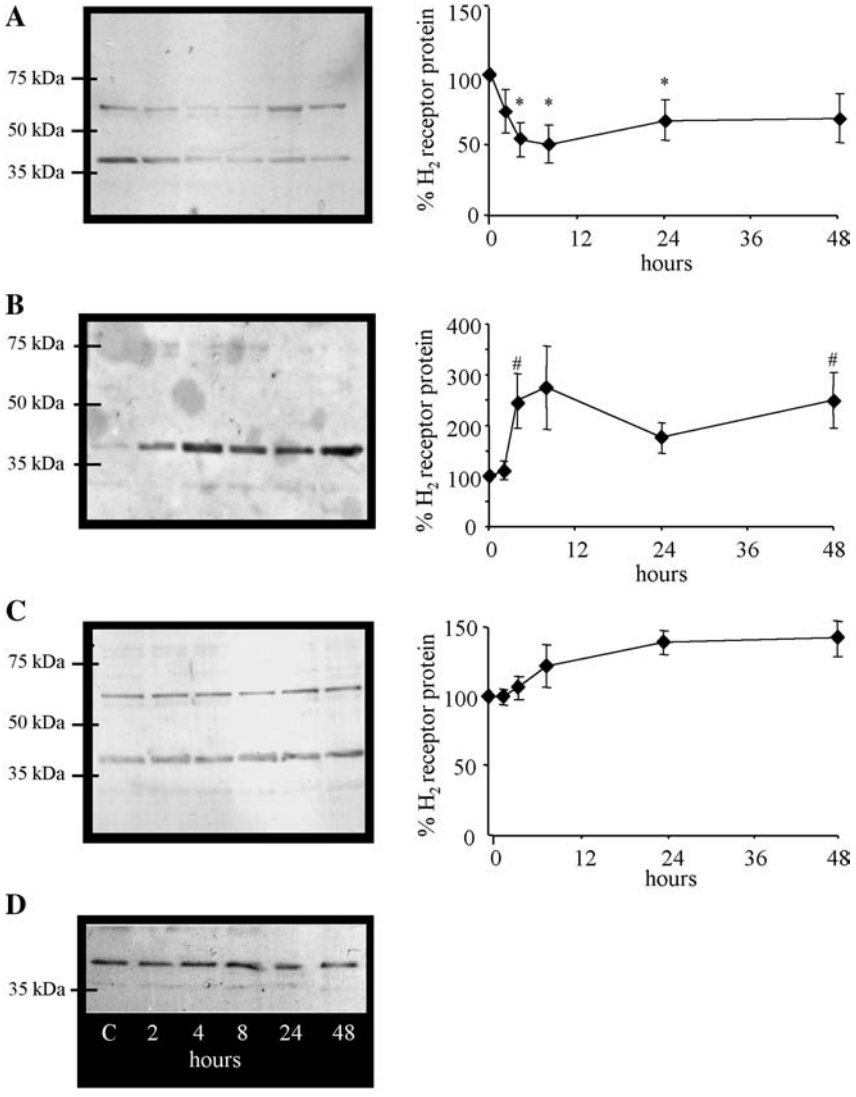

Figure 4. Time-dependent effect of histamine on H_2 receptor localization. HUVECs were stimulated with histamine (1 mM) for 2–48 h. Membrane (*A*) and cytoplasmatic (*B*) fractions of HUVECs were prepared as described in Materials and methods. Fractionated samples and whole-cell lysate (*C*) were subjected to SDS-PAGE electrophoresis. The presence of H_2 receptor protein in the samples was then detected by probing respective membranes with polyclonal anti-H_2 receptor antibodies. Membranes were stripped and reprobed with an antibody against actin (*D*) to normalize experiments. Blots shown here are representative images. Results were scanned and quantified by Gel-Pro Analyser. Results in the graphs are shown as percentage modulation of H_2 receptor protein as compared to untreated HUVECs. The data represent the mean ± SE of a minimum of three independent experiments. $^{*}p < 0.02$ versus time 0; $^{\#}p < 0.04$ versus time 0 (alpha error due to multiple comparison is not considered).

significant at any time point. Smaller signals, which might represent degradation products, were not detected during histamine stimulation.

Role of NO in histamine-induced regulation of H_2 receptor expression and internalization

NO has been shown to be one of the key mediators in histamine receptor signaling. We therefore examined the role of endogenous NO in histamine-dependent H_2 receptor regulation. To assess the possible role of endogenous NO in the histamine-stimulated regulation of H_2 receptor expression, cells were treated with two different NOS isozyme inhibitors. Each agent inhibits NOS isozymes by a different mechanism. The NO inhibitor S-ethylisothiourea (ETU) is a potent and competitive inhibitor of the inducible (IC_{50} = 17 nM), endothelial (IC_{50} = 36 nM) and neuronal (IC_{50} = 29 nM) NOS isozymes at the L-arginine-binding site [37, 38]. N^G-nitro-L-arginine (L-NAME), on the other hand, is an arginine analogue which acts as a competitive and slowly reversible inhibitor of endothelial NOS (IC_{50} = 500 nM) [39]. Using agents with differing inhibition mechanisms served as a control to exclude nonspecific effects.

The incubation of endothelial cells with either ETU (1 µM) or L-NAME (50 nM) resulted in a significant time-dependent inhibition of histamine-induced down-regulation of H_2 receptor transcript (fig. 5a, compare fig. 3a). The inhibition of histamine-induced H_2 receptor mRNA down-regulation by ETU was established to be a concentration-dependent process (fig. 5a, inset). At a lower concentration, L-NAME (5 nM) did not exhibit any effects on the histamine-induced modulation of H_2 expression.

Addition of L-NAME or ETU at these concentrations to untreated cells had no effect on the regulation of H_2 receptor mRNA (data not shown). However, at higher concentrations, we observed that L-NAME (500 nM) and ETU (10 µM) induced a significant time-dependent decrease in H_2 receptor transcript in untreated endothelial cells (L-NAME: 8 h, –13.5 ± 3.1%, $p \geq 0.02$; 48 h, –33 ± 2.6%, $p \geq 0.01$; ETU, 8 h, –52.5 ± 2%, $p \geq 0.01$; 48 h, –53.4 ± 3.2%, $p \geq 0.01$).

These observations seem to indicate that the regulation of H_2 receptor mRNA expression is dependent on the cellular level of NO, where any deviation from the steady-state level of NO might induce the down-regulation of the transcript. However, at such high concentrations, L-NAME and ETU might exhibit additional as yet undefined effects on the cellular signaling cascade.

The histamine-induced reduction in cell surface expression of H_2 receptors was also significantly modulated by the inhibition of NOS inhibitors as determined by the densitometric analysis of Western blots.

Incubation of HUVECs with L-NAME or ETU at the most effective concentration (50 nM and 0.1 µM, respec-

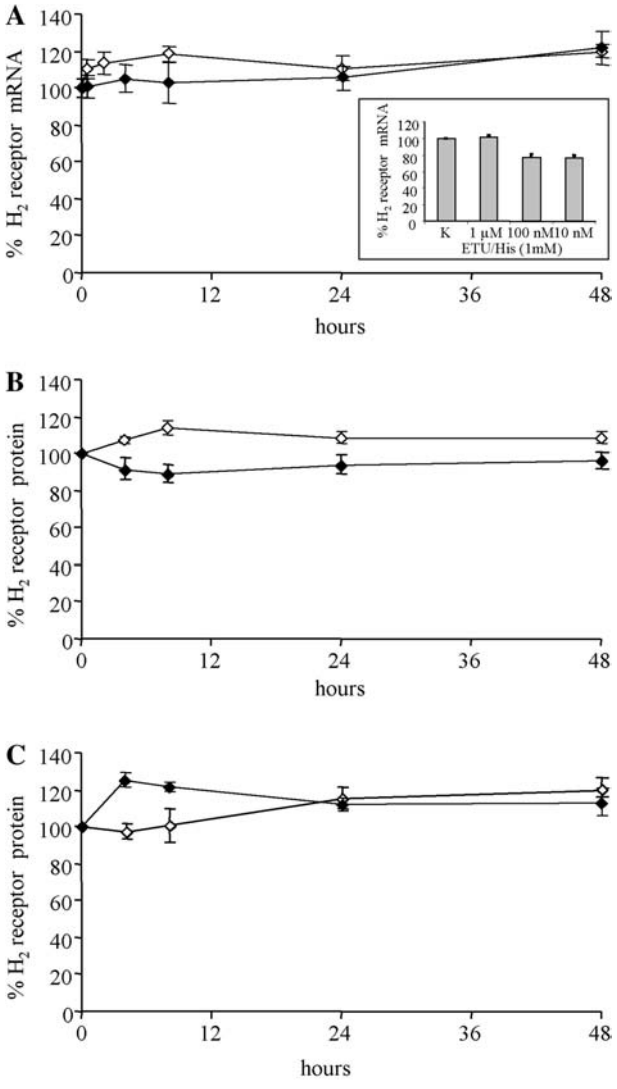

Figure 5. Role of NO in the histamine-induced modulation of H_2 receptor expression and localization. Histamine- (1 mM) stimulated HUVECs were incubated with NOS isozyme inhibitors L-NAME or ETU for 2–48 h. (*A*) RT-PCR was performed using 0.1 µg of total RNA from HUVECs. H_2 receptor and GAPDH expression levels were determined by semi-quantitative RT-PCR. RT-PCR products were quantified using HPLC. Results were normalized with respect to GAPDH. Results in the graph are shown as percentage H_2 receptor mRNA levels of the respective controls. The data represent the mean ± SE of a minimum of six independent experiments. Cell lysates of HUVECs were prepared and fractionated into membrane (*B*) and cytoplasmatic (*C*) fractions as described in Materials and methods. Aliquots were subjected to SDS-PAGE. The presence of H_2 receptor protein in the samples was detected by probing respective membranes with polyclonal anti-H_2 receptor antibodies. Results were scanned and quantified by Gel-Pro Analyser. Results in the graphs represent the percentage decrease or increase in H_2 receptor protein compared to controls. The data represent the mean ± SE of a minimum of three independent experiments; open rhombus, incubation of histamine-stimulated cells with ETU (1 µM); closed rhombus, incubation of histamine-stimulated cells with L-NAME (50 nm).

tively) during histamine (1 mM) stimulation resulted in a significant inhibition of H_2 receptor protein translocation from the cell surface into the cytoplasmic fractions during long-term stimulation of cells compared to the H_2 receptor down-regulation induced by histamine alone (fig. 5b, c; compare fig. 3a). Incubation of unstimulated HUVECs with either L-NAME or ETU at these concentrations did not exhibit an effect on the H_2 protein level in the membrane or cytoplasmatic fraction (results not shown). Our results indicate that the histamine-stimulated activation of NOS isozymes in HUVECs seems to be a key factor in the signal transduction events mediating long-term histamine-induced H_2 receptor regulation.

Discussion

Although modulation of histamine receptor subtype expression has been reported by several investigators, studies examining the molecular mechanism underlying the differential regulation of histamine receptor expression are still scarce.

In an attempt to address the question of the molecular basis for agonist-stimulated modulation of histamine receptor expression, we investigated the histamine-induced regulation of H_2 receptor subtype expression in more detail.

A prerequisite for detailed characterization of H_2 receptor regulation was the generation of polyclonal antibodies directed against an extracellular domain of the receptor protein. Results obtained by Western blot analysis, immunostaining and ELISA disclosed the polyclonal antibodies directed against the H_2 receptor to be specific and beneficial for the quantification of receptor expression.

By employing the anti-H_2 receptor antibodies we were able to show a histamine-stimulated reduction of cell surface receptors in endothelial cells that corresponded to the reduction in H_2 receptor mRNA as determined by competitive RT-PCR and reported in previous studies [10]. However, the reduction in cell surface receptor protein was comparatively delayed, with a maximum decrease after 16 h of histamine stimulation. A delayed reduction in H_2 receptor protein has also been observed when Chinese hamster ovary cells transfected with the H_2 receptor ($CHOrH_2$) were stimulated with histamine. Exposure of $CHOrH_2$ cells to 100 µM histamine resulted in an approximately 50% decrease in [^{125}I]-APT binding after 16 h [9]. H_2 receptor mRNA levels were reduced by a maximum of 70% after 4 h of histamine incubation in this study. The extent and time dependency of H_2 receptor mRNA and protein regulation were comparable to the effects observed in the present study. The comparability of receptor quantification obtained by binding studies and by applying the newly generated anti-H_2 receptor antibodies underlines the validity of our current results.

Although the time-dependency and extent of H_2 receptor down-regulation in $CHOrH_2$ cells and HUVECs are comparable, there seem to be considerable differences in the cellular mechanisms underlying the observed effects. In HUVECs, H_2 receptor reduction was determined to be regulated via the H_1 receptor [40] and a subsequent activation of NOS (see below). In $CHOrH_2$ cells, H_2 receptor down-regulation was shown to be regulated via a cAMP-dependent and cAMP-independent pathway [9]. Furthermore, CHO cells do not seem to express NOS isozymes [41].

Agonist-induced reduction of receptor levels is a well-described mechanism of GPCR regulation [for references see refs 13, 42, 43] and is considered an important feedback mechanism that prevents acute and chronic receptor over-stimulation and allows the dynamic regulation of cellular responses [12, 44]. Reduction of GPCR levels has been attributed to a combination of different processes. The rapid attenuation of receptor responsiveness, termed desensitization, has been considered to result from the internalization of cell surface receptors to intracellular membranous compartments [45–48]. Agonist-induced short-term H_2 receptor desensitization accompanied by receptor internalization has been reported previously in COS7 and HEK-293 cells transfected with HA-tagged canine H_2 receptor and Flag epitope-tagged rat H_2 receptor, respectively [49–51].

Long-term regulation of receptor density, on the other hand, has been associated with the down-regulation of the total cellular number of receptors due to a decrease in receptor mRNA and protein synthesis as well as the lysosomal degradation of preexisting receptors [52, 53].

To determine whether receptor internalization and/or degradation are mechanisms involved in the long-term reduction of cell surface levels of the H_2 receptor, we studied the cellular distribution of H_2 receptors by Western blot and densitometric analysis. Our results indicate that the slow reduction of cell surface receptor (half-maximum loss reduction = approx. 7 h) that lasts for 48 h is solely due to the translocation of the protein into the cytoplasmic fraction. This supposition is based on the observation that the reduction in cell surface receptors within the first 4 h is correlated with a continuous increase in H_2 receptor protein in the cytoplasmatic fraction, while at the same time, the overall amount of H_2 receptors in whole-cell lysate remained constant. The unvarying H_2 receptor level in whole-cell lysate might be due to the stabilization of the protein, because the H_2 receptor mRNA was significantly down-regulated during this time period. Furthermore, augmentation of mRNA expression after 8 h of histamine stimulation was paralleled by a slight increase in receptor level in whole-cell lysate during long-term stimulation.

The notion of H_2 receptor internalization as a long-term mechanism in H_2 receptor responsiveness was further

emphasized when we observed an enhanced cell surface receptor expression that was accompanied by a decrease in receptor level in the cytoplasmatic fraction after 24 h of histamine stimulation. However, cell surface receptor levels did not return to control levels even after 48 h of histamine stimulation, suggesting a new steady-state level of cell surface receptor during long-term stimulation.

The rate and extent at which GPCRs are internalized and recycled back to the plasma membrane surface are GPCR subtype specific [54–56]. The diverse patterns of GPCR desensitization and resensitization are determined by a number of factors that include receptor structure [46, 57] and a well-defined group of intracellular regulatory molecules [12, 58]. Regulatory proteins such as second messenger-dependent [59–62] and GPCR kinases [14, 63], the family of arrestins [for references see refs 11, 12, 64] and a subfamily of Ras-like small GTPases, termed Rab GTPases [65, 66] have been shown in particular to contribute to the internalization, endocytotic trafficking and subsequent recycling of GPCRs to the cell surface.

Desensitization of the H_2 receptor has been described in a variety of studies and has been linked to the expression of the GPCR kinase 2 and 3 (GRK) [14–16, 67, 68]. Furthermore, although there is no direct evidence as yet for the regulatory function of any of these molecules in the internalization of the histamine H_2 receptor, we would like to point out that a significant up-regulation of a Rab GTPase, identified as rab18, was observed when histamine-dependent gene expression in endothelial cells was analyzed by differential display [69]. In this study, we reported a continuous increase in rab18 transcripts over a period of 24 h of histamine stimulation that might provide an effective starting point for further investigation into the endocytic mechanism underlying histamine H_2 receptor internalization.

The elucidation of the endocytic mechanism resulting in H_2 receptor internalization might be of particular interest, since differences in the patterns of endocytosis are considered to translate into physiological differences of GPCR activity [12]. Furthermore, long-term GPCR internalization followed by a significant increase in cell surface receptors after 24 h of agonist stimulation as observed in this study has to our knowledge not yet been reported.

In previous studies, we were able to show that H_2 receptor mRNA down-regulation in endothelial cells is a heterologous process mediated via the activation of the H_1 receptor [10]. A close link of H_1 receptor stimulation, phospholipase C-mediated accumulation of cytosolic Ca^{2+} levels and the activation of Ca^{2+}-dependent signal transduction enzymes, such as endothelial NOS has been shown in a wide variety of studies [for reference see ref. 70]. Our own results underline the association of the H_1 receptor with these previously delineated signal transduction events, since H_1 receptor-mediated processes such as adhesion of neutrophils [19], mediator release [unpublished observations] and the regulation of gene expression of the transcription factor HZF2 [18] were shown to be stimulated within minutes via activation of NOS isozymes. This evidence indicating NO as an important signal transduction molecule in H_1 receptor-mediated cellular responses was further emphasized by our current results showing a distinct role of NO in the histamine-induced modulation of H_2 receptor expression and distribution, as determined by the inhibition of NOS isozymes by two different NOS isozyme inhibitors (L-NAME and ETU). Although there have been no studies associating this second messenger with the regulation of GPCR internalization, there is strong evidence for the NO-dependent regulation of GPCR gene expression [31, 71–73].

The long-term loss of H_2 receptor protein from the cell surface might indicate a histamine-induced shift toward H_1 receptor-mediated endothelial cell responses. This supposition is underlined by our previous results showing that H_1 receptor mRNA is only marginally down-regulated for a short period of time during histamine stimulation in endothelial cells [10]. H_1 receptor-mediated responses in endothelial cells include the ability of histamine to directly cause vasodilation [74], increased vascular permeability [75], the adhesion of neutrophils [19, 76, 77] and the release of stored vasoactive factors such as interleukin-8 [78] and P-selectin [76]. Therefore, one can reasonably conclude that long-term internalization of H_2 receptor protein might promote an enhanced histamine-induced inflammatory response by endothelial cells. This assumption is further emphasized by our previous studies showing that histamine-induced H_1-mediated adhesion of neutrophils to endothelial cells was decreased when forskolin, a cAMP-inducing substance, was added [19]. Moreover, the adhesion of neutrophils to unstimulated endothelial cells was significantly increased when the production of cAMP was blocked by the addition of an adenylate cyclase inhibitor, indicating an inhibitory effect of cAMP on neutrophil adhesion in unstimulated cells. The stimulation of the H_2 receptor subtype is classically associated with the activation of adenylate cyclase and a subsequent accumulation of intracellular cAMP [70]. Hence long-term down-regulation of the H_2 receptor would prevent such a protective antiinflammatory feedback mechanism mediated via this receptor subtype in endothelial cells.

A negative feedback regulation mediated via the H_2 receptor in a wider variety of pathologies has been suggested by studies examining histamine-dependent T cell responses. Histamine acting through the H_1 receptor enhanced T helper 1 (T_H1) cell responses, whereas T_H1 and T_H2 responses are suppressed by signaling via H_2 receptors [79, 80]. Furthermore, the H_1 receptor was recently identified as a disease locus (Bphs) associated with au-

toimmune T cell and vascular responses regulated by histamine (VAASH) after pertussis toxin sensitization [81]. This study demonstrated that *Hrh1*$^{-/-}$ mice were completely resistant to VAASH, whereas *Hrh2*$^{-/-}$ mice were fully susceptible. Moreover, the T cell response in *Hrh1*$^{-/-}$ mice was strongly T_H2 biased and associated with less severe disease.

Our results indicate that the long-term modulation of H_2 receptor distribution is due to internalization of the protein. The long-term internalization is an intriguing aspect of receptor regulation, since it may provide a means for the flexible regulation of cellular responses that might have to be taken into consideration for long-term therapeutic interventions in progressing inflammatory conditions such as sepsis or allergy. The effect of antagonist blocking of H_1 receptor-mediated responses during these conditions may feasibly be surpassed by simultaneous agent-induced rapid protein synthesis-independent stimulation of H_2 receptor relocation to the cell surface and thereby restoration of feedback mechanisms.

Furthermore, recent findings indicating that GPCR desensitization and endocytosis might result in the coupling of GPCRs to alternative signal transduction pathways [82, 83] could revolutionize our understanding and thereby the potential of manipulating GPCR function.

However, any conjecture concerning therapeutic interventions based on our results can only be highly speculative, since the regulation of H_2 receptor expression as well as H_2 receptor-associated protective feedback mechanisms during inflammatory conditions such as sepsis and allergy have yet to be elucidated in vivo.

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