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Transcriptional down-regulation of human α_{2A}-adrenoceptors by IFNγ and TNFα in intestinal cells

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ABSTRACT

α_{2A}-adrenoceptors are expressed on intestinal cells and they participate in the control of epithelial functions such as solute and water transport or cell proliferation. In pathological conditions, pro-inflammatory cytokines secreted by lymphocytes are responsible for modification of intestinal cell characteristics including phenotype switch and changes in the expression of pumps and ion channels. Using the HT29 cell line as a model, the present work examined the effect of two inflammatory cytokines, interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), on the expression of the human α_{2A}-adrenoceptor. Exposure of cells to either IFNγ or TNFα resulted in a concentration- and time-dependent diminution of [^{3}H]RX821002 binding sites, which is preceded by a large decrease in the amount of α_{2A}-adrenoceptor mRNA. The cytokines did not affect the receptor mRNA half-life, but inhibited the activity of a luciferase construct containing the promoter region of α_{2A}-adrenoceptor gene, indicating that a decrease in the transcription rate is primarily responsible for the diminution of receptor expression. Exposure of cells to either IFNγ or TNFα caused increased production of reactive oxygen species and transient phosphorylation of extracellular signal-regulated kinase (Erk1/2). The effect of cytokines was mimicked by H_2O_2 but was unaffected by the addition of anti-oxidants. The blockade of Erk1/2 activation by PD98059 blunted the effect of TNFα but not of IFNγ. In conclusion, the present findings demonstrate that IFNγ and TNFα diminish the α_{2A}-adrenoceptor expression in HT29 cells by decreasing the transcription rate without modifying the stability of mRNA. The transcription inhibition is however triggered via different signalling pathways. The results suggest that cytokine-mediated down-regulation of α_{2A}-adrenoceptor could contribute to the pathogenesis of inflammatory bowel disease.

1. Introduction

α_2-adrenoceptors are G-protein coupled receptors which are widely distributed throughout the body and which are involved in a large panel of physiological processes such as regulation of blood pressure, anti-lipolysis or inhibition of insulin secretion (Brede et al., 2004; Ruffolo et al., 1993). Pharmacological and molecular studies carried out on different species, including human, have shown that α_2-adrenoceptors of the A subtype (α_{2A}-adrenoceptors) are expressed on intestinal epithelial cells, especially in the crypt compartment (Valet et al., 1993). According to *in vivo* studies, these receptors are responsible for major effects of catecholamines on intestinal functions including stimulation of Na^+ and H_2O absorption, inhibition of Cl^- and HCO_3^- secretion (Chang et al., 1983; Liu and Coupar, 1997) and promotion of epithelial cell proliferation (Tutton and Barkla, 1987). On the basis of *in vitro* experiments on human colon cancerous cell lines, α_2-adrenoceptors were also proposed to increase the activity of the peptide transporter pepT1 (Berlioz et al., 2000) and to accelerate cell proliferation and migration (Schaak et al., 2000; Buffin-Meyer et al., 2007).

The activity of G-protein coupled receptors is under the control of a set of timely-operated phenomena including receptor desensitization, receptor internalization and receptor down-regulation (von Zastrow, 2001, 2003). Desensitization classically proceeds via receptor phosphorylation, followed by its interaction with arrestins, which uncouples receptors from G proteins and results in signal disruption. Down-regulation usually involves the endocytic trafficking; it consists in receptor internalization, intracellular sorting and degradation. Thus, in contrast to desensitization, down-regulation is associated with a decreased density of cell surface receptors. Another way to modulate G-protein coupled receptor density is achieved by change in gene transcription, alteration of mRNA stability or modification of translation rate. The above-mentioned changes can be induced not only by

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specific agonists of the considered receptor (homologous regulation) but also by agonists of other receptors (heterologous regulation). Homologous and heterologous regulation of α_{2A}-adrenoceptor has been studied in different cell systems, including the human colon cancerous cell-line HT29. In these cells, exposure to norepinephrine caused both desensitization and down-regulation (Jones et al., 1990). Furthermore, the expression of α_{2A}-adrenoceptor increased upon exposure to vasoactive intestinal peptide or cAMP (Sakaue and Hoffman, 1991) whereas it decreased after treatment with insulin, epidermal growth factor (Devedjian et al., 1991) or short chain fatty acids (Devedjian et al., 1996).

There is now clear evidence that interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), released by cells from the immune system, play a key role in the pathogenesis of inflammatory bowel diseases (Papadakis and Targan, 2000). Experiments carried out on different animal models and on cultured epithelia have shown that these two pro-inflammatory cytokines exert their deleterious effects by different means including disruption of tight junctions, induction of enterocyte apoptosis, switch of epithelial cell phenotype to antigen-presenting cells, and changes in the expression of several genes and their transcription factors. Other studies but in non-intestinal cells have also demonstrated that cytokines can also change the expression or the function of G-protein coupled receptors. Examples of receptors which are affected upon exposure to IFNγ or TNFα include adenosine A_{2A} and A_{2B} receptors (Khoa et al., 2001; Nguyen et al., 2003; Trincavelli et al., 2002), bradykinin B_1 and B_2 receptors (Phagoo et al., 1997; Schanstra et al., 1999) as well as β_1-, β_2- and β_3-adrenoceptors (Hadri et al., 1997). To our knowledge, no study has examined the effects of cytokines on α_2-adrenoceptor expression. Nevertheless some evidences argue in favour of a possible regulation by cytokines in gut. In experimental colitis induced by intrarectal administration of 2,4-dinitrobenzenesulphonic acid to rats, Blandizzi et al. (2003) observed an increased α_{2A}-adrenoceptor expression in both ileal and colonic muscular layers, without concomitant change in mucosal tissues. Furthermore, an increased density of α_2-adrenergic binding sites was detected in cell membrane preparations obtained from jejunal muscular tissues of guinea-pigs with small bowel inflammation (Martinolle et al., 1993).

The aim of the present work was to investigate the effects of IFNγ and TNFα, on the expression of α_{2A}-adrenoceptor in cells from intestinal epithelium origin. Experiments were performed in HT29 cells. Previous studies of this human colon adenocarcinoma cell line have demonstrated that it expresses α_2-adrenoceptors (Bouscarel et al., 1985) as well as receptors for the two cytokines (Crotty et al., 1992; Panja et al., 1998). We demonstrate that exposure of HT29 cells to IFNγ or TNFα induces a concentration- and time-dependent decrease of α_2-adrenoceptor density. This effect is correlated with a decreased amount of receptor mRNA due to an attenuation of transcription rate. The cellular mechanisms responsible of these effects were also investigated.

2. Materials and methods

2.1. Drugs and reagents

α[^{32}P]-UTP (800 Ci/mmol) was from ICN (Costa Mesa, CA). The mouse anti phosphorylated-Erk1/2 monoclonal antibody, rabbit anti-Erk2 polyclonal antibody and horseradish peroxidase-conjugated goat anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). The horseradish peroxidase-conjugated donkey anti-rabbit IgG, nitrocellulose membranes, ECL Western blotting system and [^{3}H]RX821002 ([^{3}H]-2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1*H*-imidazole, 59 Ci/mmol) were from Amersham Biosciences (Little Chalfont, UK). PD98059 (2′-amino-3′-methoxyflavone) was obtained from Calbiochem (La Jolla, CA). Fetal calf serum was purchased from Gibco-BRL (Cergy Pontoise, France). Phentolamine was donated by Ciba-Geigy (Basel, Switzerland). Human recombinant tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ), actinomycin D, *o*-nitrophenyl β-D-galactopyranoside, hydrogen peroxide (H_2O_2), *N*-acetyl cysteine, pyrrolidine dithiocarbamate, phenyl-*N*-*tert*-butylnitrone, lazaroid U83836E and NG-nitro-L-arginine methyl ester were from Sigma (St Louis, MO). T3 RNA polymerase, TFX-50 transfection reagent, and luciferase assay reagents came from Promega (Madison, WI).

2.2. Cell culture

The HT29 cell line was routinely cultured in Dulbecco's modified Eagle's medium, containing 25 mM glucose, 100 μg/ml streptomycin, 100 U/ml penicillin and supplemented with 5% heat inactivated fetal calf serum. Unless otherwise indicated, all experiments were carried out on post-confluent cells deprived of serum for 24 h.

2.3. Receptor quantification

Frozen cells were harvested in 50 mM Tris–HCl buffer (pH 7.5) containing 5 mM EDTA and centrifuged at 27,000 g for 10 min at 4 °C. The pellet was taken up in TM buffer (50 mM Tris–HCl, 0.5 mM $MgCl_2$, pH 7.5) and centrifuged again. The final pellet was suspended in the appropriate volume of TM buffer and immediately used for binding experiments. Briefly, membranes were incubated at 25 °C in a 400 μl final volume of TM buffer containing [^{3}H]RX821002. After a 45 min period of incubation, membrane bound radioactivity was separated from free by rapid filtration through a Whatman GF/C filter. Retained radioactivity was counted by liquid scintillation spectrometry and specific binding was calculated as the difference between total and non-specific binding determined in the presence of 10^{-5} M phentolamine. Saturation experiments were performed in the presence of [^{3}H]RX821002 concentration ranging from 0.25 to 8.5 nM. Values of Bmax and K_D were calculated by computer-assisted analysis of the data using GraphPad Prism (GraphPad Software, San Diego, CA).

2.4. RNA extraction and RNase protection assay

Cellular RNA was extracted using the guanidinium isothiocyanate/phenol-chloroform method (Chomczynski and Sacchi, 1987) and the riboprobes for quantification of α_{2A}-adrenoceptor and β-actin mRNAs were generated as previously described (Cayla et al., 1999; Devedjian et al., 1991). Briefly, the plasmids pBlueScript KS+ (Statagene, La Jolla, CA), containing either a 352-base fragment corresponding to nucleotides 1041–1392 of the α_{2A}-adrenoceptor gene or a 236-base fragment corresponding to nucleotides 415–650 of the β-actin cDNA (exon 3), were linearized with the appropriated restriction enzyme and the [^{32}P]-labelled antisense RNAs were synthesized in using T3 RNA polymerase. RNase protection assays were performed as described previously (Schaak et al., 1997). Lyophilized RNA were taken up in 30 μl of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM Pipes, pH 6.7) containing an excess of [^{32}P]-labelled riboprobe. The samples were heated to 95 °C for 5 min and then placed at 55 °C for 14 h. Non-hybridized probe was eliminated by the addition of 0.3 ml of RNase A (40 μg/ml) and RNase T1 (2 μg/ml) in 300 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl (pH 7.5). After 2 h at 37 °C, digestion was stopped by addition of 5 μl of proteinase K (10 mg/ml) and the samples were further incubated for 15 min at 37 °C. Carrier tRNA (10 μg) and 0.3 ml of solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol and 0.5% sarkosyl) were added to each tube and protected hybrids were precipitated with isopropyl alcohol. Pellets were washed with 70% ethanol, air-dried, taken up in sample buffer (97% deionized formamide, 0.1% SDS, 10 mM Tris–HCl, pH 7.0) and run on a 5% polyacrylamide gel containing 7 M urea. Gels were fixed, dried and exposed for 48 h at −80 °C to X-ray film for autoradiography.

2.5. DNA constructs, cell transfection and reporter gene assays

The plasmid pGL3-α_{2A}-promoter, which contains the promoter region of α_{2A}-adrenoceptor gene (nucleotides −2076/+4 relative to translation start) in fusion with the luciferase gene, was constructed as follow. The HPalpha2GEN clone (American Type Culture Collection, clone number 59302), which contains a genomic 5.5 kb BamHI/BamHI fragment encompassing the α_{2A}-adrenoceptor gene (Kobilka et al., 1987), was digested with either BamHI and NheI or NheI and NcoI. The fragments of interest (respectively 1.9 and 0.2 kb) were purified and ligated into the BglII and NcoI sites of pGL3-Basic vector (Promega, Madison WI). HT29 cells were seeded in 6 well plates at the density of 10^6 cells per well. The following day, they were rinsed with PBS and transfected with a mixture containing pGL3-α_{2A}-promoter (2 μg), pCMV-LacZ (0.5 μg) and TFX-50 reagent (7.5 μl) in 400 μl of serum-free culture medium. After 4 h at 37 °C, 2.5 ml of complete medium were added to each well. One day post-transfection, cells were placed in fresh medium and treated or not with IFNγ or TNFα for 24 h. They were then rinsed with PBS, harvested and luciferase and β-galactosidase activities were measured using luciferase assay reagent and *o*-nitrophenyl β-D-galactopyranoside as substrates.

2.6. Measurement of H_2O_2 production

H_2O_2 production was measured by chemiluminescence assay in the presence of luminol (10 μM) and horseradish peroxidase (0.1 U/ml), as previously described (Pizzinat et al., 1999). Chemiluminescence was monitored during 60 min at 37 °C, using a luminometer (Bio-Orbit 1251, Turku, Finland) and the area under the curve (total chemiluminescence emission) was calculated by the Bio-Orbit MultiUse program.

2.7. Immunodetection of Erk1/2

Cells were rapidly rinsed with ice-cold PBS and harvested in 1 ml of Tris–HCl buffer (pH 7.4) containing 10 mM, 1% Triton-X100, 1% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM Na-orthovanadate, 1 mM PMSF and 0.5 mM aprotinin. Soluble proteins were extracted by centrifugation (15,000 g, 15 min at 4 °C), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. Phosphorylated forms of Erk1/2 were detected using an anti phosphorylated-Erk1/2 monoclonal antibody (1/2000) and revealed by chemiluminescence using the corresponding horseradish peroxidase-conjugated secondary antibody (1/10,000 goat anti-mouse IgG). In all experiments, membranes were stripped out of IgG, reprobed with anti-Erk2 polyclonal antibody (1/2000) and revealed using 1/5000 donkey anti-rabbit IgG to assess equal protein loading.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical differences between means were tested using the repeated measures ANOVA and checked for significance using Tukey's multiple comparison test. Values of $P<0.05$ were considered as statistically significant.

3. Results

3.1. IFNγ and TNFα decrease α_2-adrenoceptor density in HT29 cells

The effect of IFNγ and TNFα on α_2-adrenoceptor density was first examined by measuring [^{3}H]RX821002 binding to crude membranes prepared from HT29 cells treated for 48 h with different concentrations of each cytokine. As shown in Fig. 1A, exposure to either IFNγ or TNFα decreased [^{3}H]RX821002 binding in a concentration-dependent manner, indicating that both cytokines caused a diminution of α_2-adrenoceptor expression in these cells. This effect was significant at a concentration as low as 0.1 U/ml for IFNγ and 0.1 ng/ml for TNFα. Maximal effect was reached with 100 U/ml IFNγ and 20 ng/ml TNFα and resulted in a 65 ± 2% and 49 ± 4% reduction of binding site number, respectively. The decrease in α_2-adrenoceptors is not associated with an alteration of receptor affinity for the radioligand as no change in the dissociation constant was observed (K_D=0.85 to 1.2 nM, not shown). Results in Fig. 1B indicated that the effects of the two cytokines were also time-dependent, but with slightly different kinetics. Indeed, whereas the effect of IFNγ was rather rapid with a significant decrease in receptor density after 6 h of treatment only, the effect of TNFα became evident for periods of exposure longer than 12 h.

3.2. IFNγ and TNFα affect α_{2A}-adrenoceptor mRNA levels

The decrease in α_2-adrenoceptor density could be the result of an acceleration of its degradation or be the reflection of a decrease of its synthesis due to decreased mRNA amount. The possibility that IFNγ and TNFα reduce α_2-adrenoceptor mRNA was therefore explored by RNase protection assay, using an antisense riboprobe derived from the gene encoding human α_{2A}-adrenoceptor. As depicted in Fig. 2, exposure of HT29 cells to either IFNγ (100 U/ml) or TNFα (20 ng/ml) reduced markedly the amount of α_{2A}-adrenoceptor mRNA. This effect appeared somewhat specific because the amount of β-actin mRNA transcripts was not significantly changed by treatments. In agreement with what previously observed for receptor density, IFNγ and TNFα exhibited a slight difference in their kinetics of action (Fig. 2B). The effect of IFNγ was very rapid, the maximum decrease of α_2-adrenoceptor mRNA being observed within 4 h of exposure to the cytokine. In comparison, TNFα acted more gradually and necessitated prolonged exposure (12 h) to reach maximum effect. As shown in Fig. 2C the effects of both cytokines on α_2-adrenoceptor mRNA were also concentration-dependent.

3.3. Mechanism of α_2-adrenoceptor mRNA decline induced by IFNγ and TNFα

Because an accelerated rate of mRNA degradation may account for the decrease of α_2-adrenoceptor mRNA, the effect of cytokines on mRNA stability was tested. For this purpose, HT29 cells were incubated with IFNγ or TNFα in the presence of actinomycin D (5 μg/ml) in order to abolish gene transcription; the disappearance of α_{2A}-adrenoceptor mRNA was then monitored over 6 h. Analysis of the data depicted in Fig. 3A, indicated that the half-life of α_{2A}-adrenoceptor mRNA in IFNγ- or TNFα-treated cells (165 ± 24 and 189 ± 15 min, respectively) was not significantly different from that in control cells (180 ± 22 min), thus suggesting that a lower rate of gene transcription may be responsible for the effects of both cytokines. To test this hypothesis, HT29 cells were transiently transfected with a plasmid containing luciferase gene under the control of the 5′ non-coding region of the α_{2A}-adrenoceptor gene. As shown in Fig. 3B, a 24 h-period of treatment with 100 U/ml IFNγ or 20 ng/ml TNFα respectively resulted in a 38% and 36% decrease in luciferase activity. Taken together, these results strongly suggest that receptor down-regulation promoted by IFNγ and TNFα is due to a decreased in the transcription rate of α_{2A}-adrenoceptor gene.

3.4. Signalling pathway mediating cytokines effect

Finally, the signalling pathways whereby IFNγ and TNFα decrease the α_{2A}-adrenoceptor expression were investigated. Since reactive oxygen species were recognized to act as intracellular mediators of IFNγ and TNFα action, their implication was tested. As demonstrated by the use of a chemiluminescence method allowing to measure intracellular content of hydrogen peroxide (H_2O_2) in intact cells (Fig. 4A), a 24 h-period of treatment with 100 U/ml IFNγ or 20 ng/ml TNFα significantly increased H_2O_2 levels. Moreover, a 24 h-exposure of HT29 cells to exogenous H_2O_2, resulted in a concentration-dependent decrease in α_2-adrenoceptor density (Fig. 4B). This effect was correlated

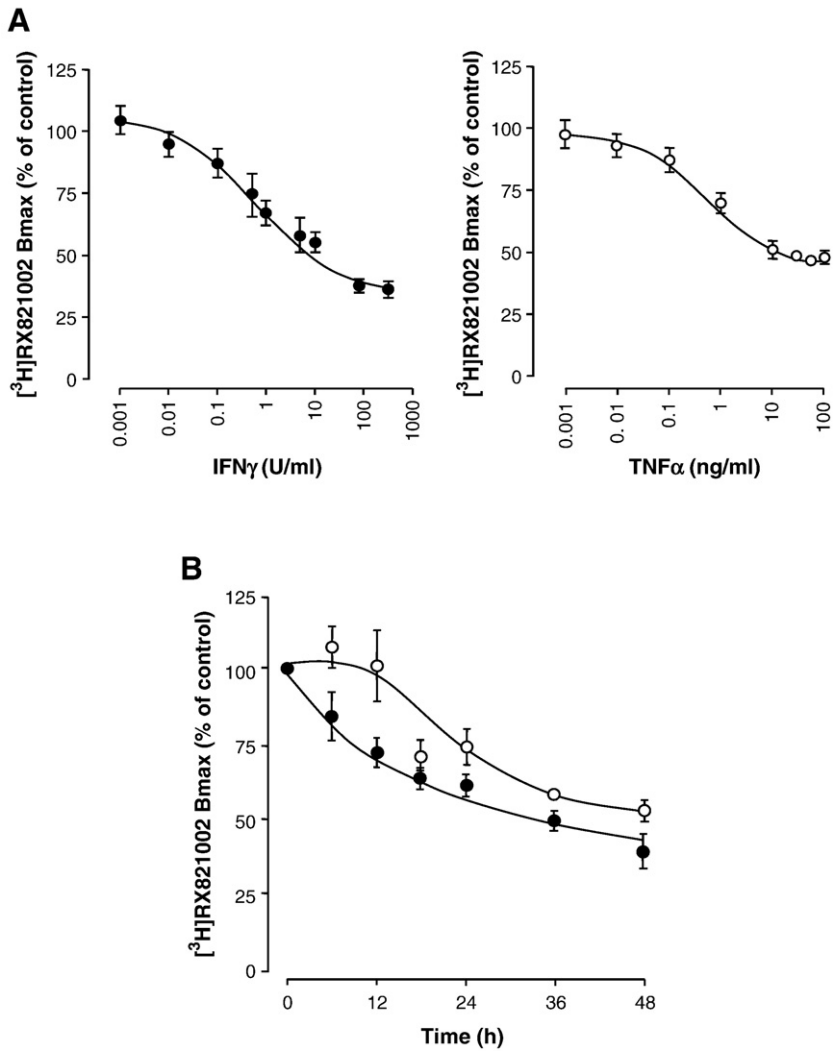

Fig. 1. IFNγ and TNFα decrease α_2-adrenoceptor density. (A) Concentration-dependent effect of IFNγ and TNFα: Serum-deprived HT29 cells were incubated for 48 h with increasing concentrations of IFNγ (left panel) or TNFα (right panel). (B) Kinetics of IFNγ and TNFα action: Serum-deprived HT29 cells were incubated with 100 U/ml IFNγ (black symbol) or 20 ng/ml TNFα (white symbol) for different period of times. In (A) and (B), cell membranes were prepared and α_2-adrenoceptor density was determined by binding studies using [^{3}H] RX821002. The number of [^{3}H]RX821002 binding sites (Bmax) was expressed as percentage of that in untreated cells. Results are means ± S.E.M. from three independent experiments.

to dramatic decrease of α_{2A}-adrenoceptor mRNA and was not due to an increased cellular mortality (not shown). However, inconsistent with a role of reactive oxygen species, cell pre-treatment with 20 mM of *N*-acetyl cysteine did not impair the incidence of IFNγ or TNFα on α_2-adrenoceptor expression (Table 1). Similar results were obtained in the presence of other anti-oxidants such as 100 μM pyrrolidine dithiocarbamate, 500 μM phenyl-*N*-*tert*-butylnitrone or 20 μM lazaroid U83836E. Therefore, although reactive oxygen species decrease the expression of the α_{2A}-adrenoceptor in HT29 cells, they are probably not involved in the effect of IFNγ or TNFα. The implication of nitric oxide (NO) was also investigated, because this messenger has been associated with the initiation and maintenance of inflammation in human inflammatory bowel disease (Kolios et al., 2004). Similarly to what found with H_2O_2, exposure of HT29 cells to the NO donor, sodium nitroprussiate (100 μM), resulted in a 35% decrease of α_{2A}-adrenoceptor density. However, the addition of the NO synthase inhibitor (NG-nitro-L-arginine methyl ester, 1 mM) did not reverse the cytokine effect (Table 1). It is known that cytokines can activate extracellular signal-regulated kinases (Erk1/2). Therefore the possibility that this pathway mediates IFNγ or TNFα effects was tested. As depicted in Fig. 5A, IFNγ and TNFα induce a rapid and transient increase of Erk1/2 phosphorylation, which peaked 15 min after the beginning of the treatment. The implication of Erk1/2 in the effect of cytokines on α_{2A}-adrenoceptor expression was then examined by using PD98059, a specific inhibitor of mitogen-activated protein kinase kinases (MEK) which abolishes Erk1/2 activation. Results presented in Fig. 5B indicated that a 30 min preincubation with PD98059 blunted the effect of TNFα but did not affect that of IFNγ. Therefore, the effect of TNFα on the expression of α_2-adrenoceptor appears to be mediated by Erk1/2 activation, while the effect of IFNγ is triggered by another mechanism.

4. Discussion

In intestinal mucosa, epithelial cells are in close contact with cells from the lymphoid and myeloid lineage, which are able to produce cytokines. Numerous studies carried out on patients or animal models have shown that the production of pro-inflammatory cytokines, such as IFNγ and TNFα, is highly increased in inflammatory bowel disease (Fuss, 2003; Papadakis and Targan, 2000; Wittig and Zeitz, 2003). The present work demonstrates that IFNγ and TNFα effectively reduce the expression of the α_{2A}-adrenoceptor in HT29 cells. The effect of both cytokines is concentration- and time-dependent, and is detected both at mRNA and protein levels. The concentration of IFNγ promoting half-maximal effect is 2.2 U/ml (equivalent to 0.22 ng/ml) a value that is in the range of the circulating concentrations of this cytokine in inflammatory situations. Indeed, the seric level of IFNγ was found to

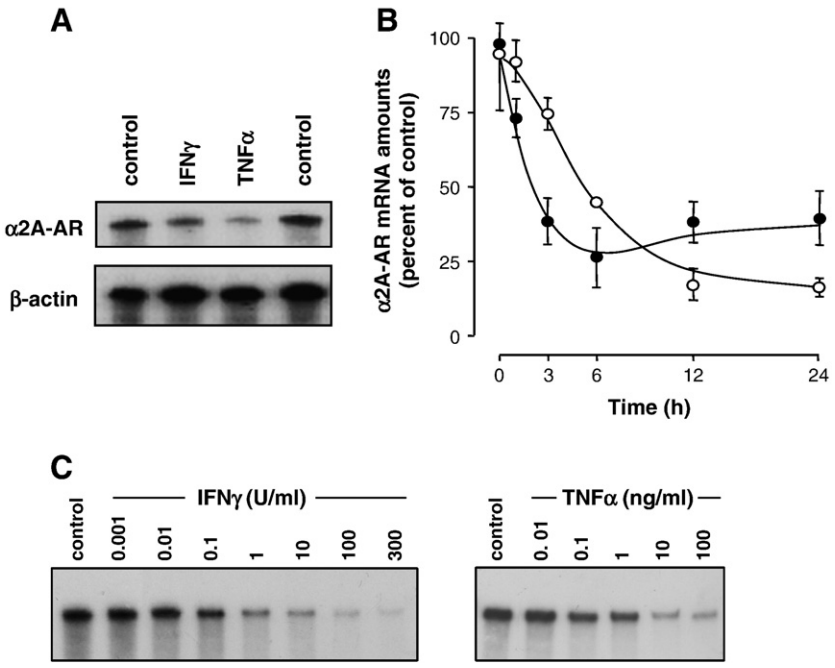

Fig. 2. IFNγ and TNFα decrease α_{2A}-adrenoceptor mRNA levels. Serum-deprived HT29 cells were treated with either IFNγ or TNFα. Cellular RNAs were extracted and the amounts of α_{2A}-adrenoceptor mRNA and β-actin mRNA (taken as internal standard) were measured by RNase protection assay using specific riboprobes. (A) Autoradiogram of a typical RNase protection assay: In this specific experiment HT29 cells were exposed for 12 h to 100 U/ml IFNγ, 20 ng/ml TNFα or vehicle (control). The amounts of α_{2A}-adrenoceptor mRNA (upper panel) and β-actin mRNA (lower panel) were determined using corresponding riboprobes. (B) Time-course of the change in α_{2A}-adrenoceptor mRNA: HT29 cells were exposed for different periods of time to 100 U/ml IFNγ (black symbol) or 20 ng/ml TNFα (white symbol). At each experimental point, the amounts of α_{2A}-adrenoceptor and β-actin mRNAs were determined by RNase protection assay and densitometric analysis of the autoradiograms. The amounts of α_2-adrenoceptor mRNA were normalized by β-actin and then expressed as the percent of that in untreated cells. Results are means±S.E.M. from three independent experiments. (C) The effects of cytokines on α_{2A}-adrenoceptor mRNA amount are concentration-dependent: HT29 cells were incubated for 6 h with increasing concentrations of IFNγ (left panel) or TNFα (right panel) and the amounts of α_{2A}-adrenoceptor mRNA were determined as in panel A.

be 0.63 ng/ml in children with food allergy (Hofman, 1995) and to reach concentration as high as 20 μg/ml in rats treated with trinitrobenzenesulfonic acid (Dasgupta et al., 2001). Half-maximal effect of TNFα was observed at 1 ng/ml. Such a concentration is also relevant in the colon, as TNFα concentration in stool from normal children is around 60 pg/g stool but attains 1 ng/g in Crohn's disease (Braegger et al., 1992). The modulation of intestinal α_2-adrenoceptor expression was already studied in experimental models of inflammatory bowel disease such as guinea-pigs treated with trinitrobenzenesulfonic acid (Martinolle et al., 1993) and rats treated with 2,4-dinitrobenzenesulphonic acid (Blandizzi et al., 2003). A significant increase in [^{3}H]-rauwolscine binding sites was found in smooth-muscle membrane preparations from guinea-pig jejunum (Martinolle et al., 1993). Similarly, an increase in the α_2-adrenoceptor mRNA measured by RT-PCR was found in the muscular layer from ileum and colon of 2,4-dinitrobenzenesulphonic acid-treated rats. By contrast, no change was observed when mucosal compartment was considered (Blandizzi et al., 2003). Beside the fact that the purity of mucosa scraped from inflamed gut is questionable; the apparent discrepancy between this later result and ours may result from the fact that the present work investigated the effects of two cytokines, IFNγ and TNFα, on a simplified *in vitro* model, while previous studies were conducted in more complex animal models in which levels of other cytokines, such as interleukins 1, 5, 6, 8 and 12, are also affected (Papadakis and Targan, 2000) and in which sympathetic innervation of the inflamed mucosa is partially lost (Straub et al., 2006).

In our study, the decrease in receptor number after IFNγ or TNFα treatment is preceded and correlated with reduced mRNA steady-state levels and the delay between mRNA reduction and protein decrease is consistent with a α_{2A}-adrenoceptor half-life of 26 h in HT29 cells (Paris et al., 1987). The combined use of actinomycin D and of a luciferase construct containing the promoter region of α_{2A}-adrenoceptor gene provided some insight into the mechanisms whereby the two cytokines decrease the steady-state level of α_{2A}-adrenoceptor mRNA. Indeed, the determination of the receptor mRNA half-life indicates that the decrease in receptor mRNA is not due to an accelerated rate of its degradation, but results from an attenuated rate of gene transcription. A change in the expression of various G-protein coupled receptors has been reported in several models of inflammatory diseases. For example, a marked decrease in angiotensin AT_1 receptor expression was observed in all organs of septic rats (Bucher et al., 2001). Similarly, α_1-adrenoceptors are down-regulated in several organs (including heart, aorta and lung) after injection of lipopolysaccharide to rats (Bucher et al., 2003). TNFα and IFNγ were also shown to decrease the amount of vasopressin V_{1A} receptor mRNA and vasopressin binding in rat hepatocytes (Bucher et al., 2002). An increase in the number and an augmentation of the functionality of adenosine A_{2A} and A_{2B} receptors were noticed in the human monocytic cell line THP-1 treated with TNFα, while a down-regulation of adenosine A_{2A} receptor was found in the same cells treated with IFNγ (Khoa et al., 2001). Recently, Khoa et al. (2006) showed that treatment of THP-1 cells with TNFα decreases G-protein coupled receptor kinase 2 expression and inhibits adenosine A_{2A} receptor desensitization, indicating that pro-inflammatory cytokines may modulate cellular signalling and modify density of G-protein coupled receptors by multiple mechanisms. The use of high-density microarrays on biopsies taken from the sigmoid colon mucosa has recently allowed comparative examination of the transcriptome in patients suffering Crohn's disease or ulcerative colitis (Costello et al., 2005). No significant change in the level of α_{2A}-adrenoceptor transcript was reported in this study, but a decrease in norepinephrine transporter expression was observed in patients with Crohn's disease. According to other studies in mice with dextran sodium sulphate-induced colitis as well as in patients with Crohn's disease (Straub et al., 2005, 2006), the decrease of the norepinephrine transporter is likely the consequence of a loss of sympathetic nerve fibres in the mucosa and submucosa. One may therefore speculate that a diminution of

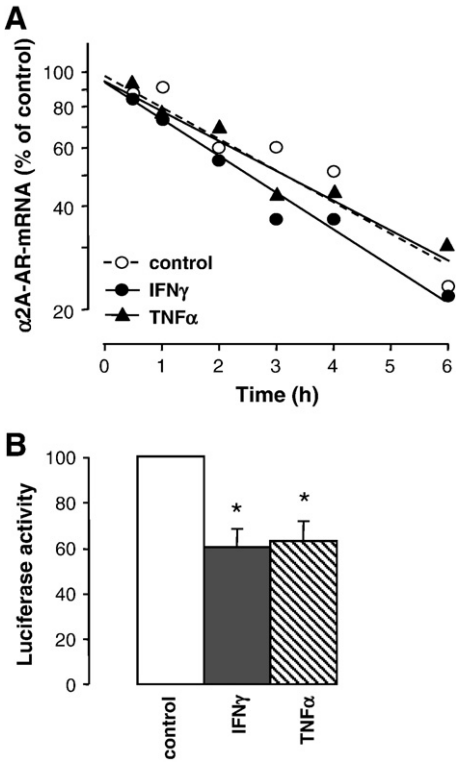

Fig. 3. Mechanism of IFNγ and TNFα action. (A) Effect of cytokines on α_{2A}-adrenoceptor mRNA half-life: Serum-deprived HT29 cells were placed in medium containing 5 μg/ml actinomycin D (white circle), 5 μg/ml actinomycin D+100 U/ml IFNγ (black circle) or 5 μg/ml actinomycin D+20 ng/ml TNFα (black triangle). Cells were harvested at indicated time, cellular RNAs were extracted, and the amounts of receptor transcript measured by RNase protection assay. Amounts of α_{2A}-adrenoceptor mRNA are expressed as percent of that in control cells at zero time. Results are means from two independent experiments and were fitted by least-square linear regression analysis. (B) Effect of cytokines on the transcriptional activity of the promoter region of the α_{2A}-adrenoceptor gene: HT29 cells were co-transfected with pCMV-LacZ and pGL3-α_{2A}-promoter, a plasmid containing the promoter region of α_{2A}-adrenoceptor gene in fusion with luciferase. One day post-transfection, cells were placed in fresh medium and exposed to 100 U/ml IFNγ or 20 ng/ml TNFα. Activity of the two reporter genes was measured after 24 h of treatment, luciferase activity was normalized by β-galactosidase and expressed as percentage of that in untreated cells (control). Results are means±S.E.M. from four independent experiments. *$P<0.01$, compared to control.

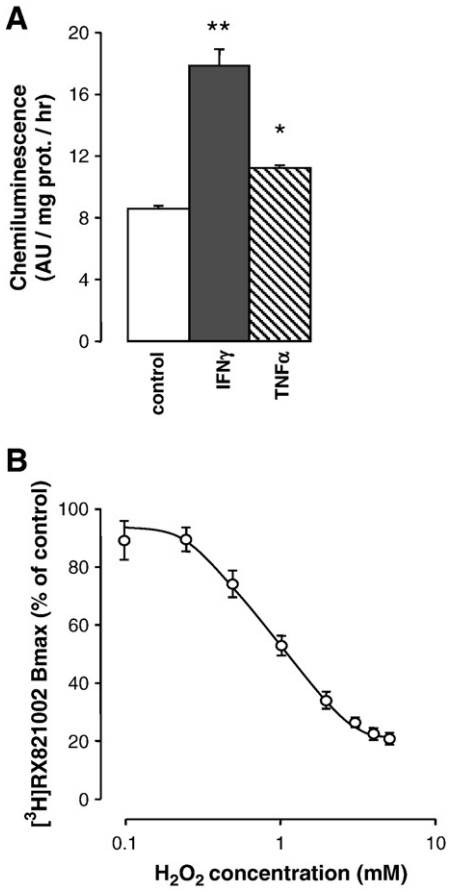

Fig. 4. Effect of H_2O_2 on α_2-adrenoceptor expression. (A) Cell exposure to IFNγ or TNFα increases H_2O_2 production: Serum-deprived HT29 cells were incubated for 24 h with 100 U/ml IFNγ or 20 ng/ml TNFα and H_2O_2 production was measured in intact cells by chemiluminescence. The light emission was monitored during 60 min and the area under the curve quantified. Results are means±S.E.M. from three independent experiments. *$P<0.01$ and **$P<0.001$, compared to control. (B) Cell exposure to H_2O_2 decreases α_2-adrenoceptor expression: Serum-deprived HT29 cells were incubated with increasing concentrations of H_2O_2. After a 24 h-period of treatment, cells were harvested, membranes were prepared and the density of α_2-adrenoceptors was estimated by analysis of [^{3}H]RX821002 binding saturation isotherms. The number of [^{3}H]RX821002 binding sites (Bmax) was expressed as percentage of that in untreated control cells. Results are means±S.E.M. from three independent experiments.

epithelial α_{2A}-adrenoceptor expression consecutive to cytokine raise combined with the decrease of neurotransmitter level consecutive to sympathetic fibre loss may result in a significant reduction of α_2-adrenergic signalling in intestinal epithelium.

As suggested by the difference in the kinetics of their effects on the receptor protein and on its mRNA, the present work also indicates that the two cytokines exert their effect via distinct signalling pathways. Both cytokines are able to increase intracellular level of reactive oxygen species. Moreover cell exposure to H_2O_2 decreases receptor expression. However increased production of reactive oxygen species is unlikely responsible for the effects of cytokines as none of the tested anti-oxidants was able to reverse their effects. Based on a similar approach, the implication of NO can be also excluded. TNFα having been found to activate the mitogen-activated protein kinase Erk1/2 in several cell types, including non-transformed rat jejunum crypt cells IEC-6 (Dionne et al., 1998), murine colon cell line YAMC (Kaiser et al., 1999) and HT29 cells (Jijon et al., 2002); we next investigated a possible implication of this pathway. In agreement with what previously reported, TNFα caused a rapid and transient phosphorylation of Erk1/2 in HT29 cells, moreover pre-treatment with PD98059 blunted the effect of TNFα on the α_2-adrenoceptor density, strongly suggesting that the effect of this cytokine requires Erk activation. Jijon et al. (2002) have shown that TNFα increases interleukin-8 (IL-8) secretion in HT29 cells, via a mechanism involving Erk1/2 activation and subsequent stabilization of IL-8 mRNA. The decrease in α_2-adrenoceptor expression was independent of changes in mRNA half-life, but primarily

Table 1
Effects of anti-oxidants and of L-NAME

Inhibitor	Control	IFNγ	TNFα
None	100	52±2[a]	52±2[a]
NAC	93±2	54±4[a]	51±3[a]
PDTC	97±3	42±5[a]	43±6[a]
PBN	97±6	53±11[a]	47±5[a]
Lazaroid	101±12	58±6[a]	54±6[a]
L-NAME	104±7	63±7[a]	60±3[a]

Serum-deprived HT29 cells were pre-treated for 30 min with vehicle (none), 20 mM of *N*-acetyl cysteine (NAC), 100 μM pyrrolidine dithiocarbamate (PDTC), 500 μM phenyl-*N*-*tert*-butylnitrone (PBN), 20 μM lazaroid U83836E (lazaroid) or 1 mM NG-nitro-L-arginine methyl ester (L-NAME) and then treated for 24 h with 100 U/ml IFNγ or 20 ng/ml TNFα. Cell membranes were prepared and α_2-adrenoceptor density was determined by binding studies using [^{3}H]RX821002. The number of [^{3}H]RX821002 binding sites (Bmax) was expressed as percentage of that in untreated cells. Results are means±S.E.M. from four independent experiments.

[a] $P<0.01$, significantly different from the corresponding control.

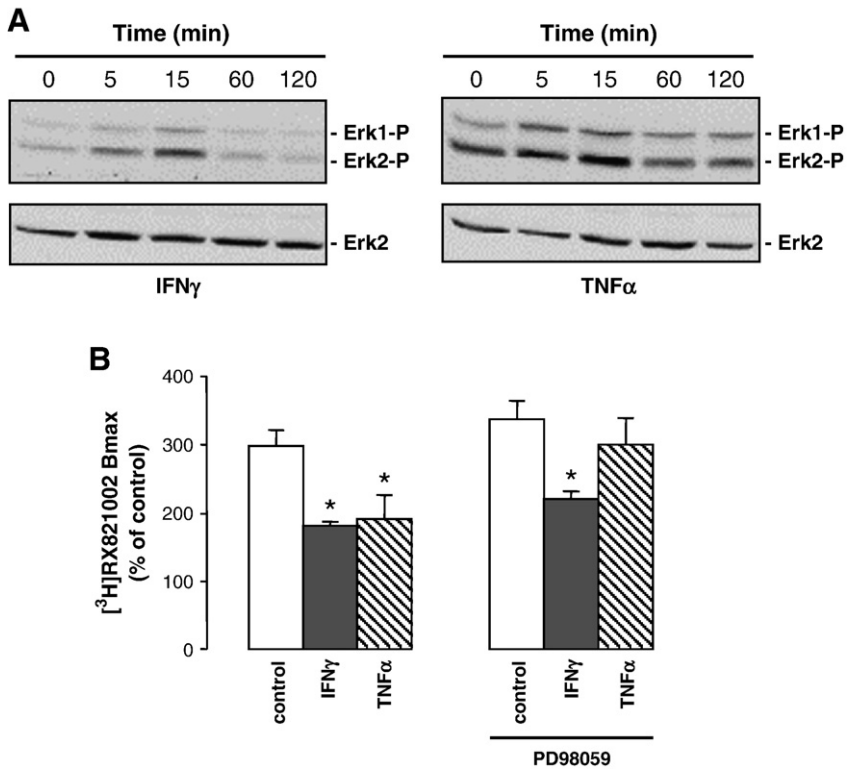

Fig. 5. Role of Erk on α_2-adrenoceptor down-regulation by cytokines. (A) IFNγ and TNFα induce Erk phosphorylation: Serum-deprived HT29 cells were incubated for different periods of time with 100 U/ml IFNγ (left panels) or 20 ng/ml TNFα (right panels). Soluble proteins were extracted, separated by gel electrophoresis and blotted onto a nitrocellulose membrane. Phosphorylated forms of Erk1/2 were detected using an anti phosphorylated-Erk1/2 monoclonal antibody (upper panels). Blots were reprobed with an anti-Erk2 antibody to assess for equal protein loading (lower panels). (B) Effect of TNFα, but not IFNγ, is dependent on Erk activation: Serum-deprived HT29 cells were pre-treated for 30 min with vehicle (right panel) or 50 μM PD98059 (right panel) and then exposed for 24 h to 100 U/ml IFNγ or 20 ng/ml TNFα. Cell membranes were prepared and α_2-adrenoceptor density was determined by binding studies using [^{3}H]RX821002. The number of [^{3}H]RX821002 binding sites (Bmax) was expressed in fmol per mg of protein. Results are means ± S.E.M. from four independent experiments. $^*P<0.01$, compared to corresponding control.

relied on a decrease of the transcription rate. Similar to TNFα, some effects of IFNγ require Erk1/2 activation. In CaCo2 cells, IFNγ reduces the activity of Na^+,K^+-ATPase and of type 1 Na^+/H^+ exchanger via a mechanism involving Erk1/2 phosphorylation (Magro et al., 2004; Magro et al., 2005). There is also phosphorylation of Erk1/2 after treatment of HT29 cells with IFNγ, however the effect of IFNγ on α_2-adrenoceptor level was insensitive to the presence of MEK inhibitor. It is now clearly established that the effects of IFNγ on gene transcription are triggered not only via the classical JAK/STAT pathway but also via STAT-independent mechanisms including PI3K/Akt and p38 MAPK pathways (Gil et al., 2001; Platanias, 2005), there is moreover accumulating evidence that coordinated activation of more than one pathway is required for generation of a given biological effect. Clarification of the mechanisms accounting for the effect of IFNγ on α_2-adrenoceptor will certainly require the combination of different approaches.

Diarrhoea is a common clinical feature of immune-mediated bowel dysfunction, and numerous *in vivo* or *in vitro* experiments indicate that IFNγ and TNFα play a key role in the changes of mucosa permeability. Effects of cytokines result from alteration of intestinal epithelium integrity as well as from modification of the expression of pumps and ion channels. For example, IFNγ markedly reduced the abundance of Na^+,K^+-ATPase in the colon of trinitrobenzenesulfonic acid-treated rats (Magro et al., 2005). In mice treated with an anti-CD3 antibody in order to induce diarrhoea, mucosal Na^+,K^+-ATPase activity was decreased and this effect was blocked by administration of an anti-TNFα antibody (Musch et al., 2002). TNFα inhibited water and Cl^- absorption and it down-regulated Na^+-K^+-$2Cl^-$ transporter and Na^+,K^+-ATPase in perfused rat distal colon (Markossian and Kreydiyyeh, 2005). In human distal colon mounted in an Ussing chamber, the addition of TNFα to the serosal compartment increased the $^{36}Cl^-$ serosal-to-mucosal flux, decreased the $^{36}Cl^-$ mucosal-to-serosal flux, and increased the ^{86}Rb net efflux, which reflects the K^+ secretion (Schmitz et al., 1996). The activation of α_2-adrenoceptors is known to stimulate Na^+ and water absorption and to inhibit Cl^- and HCO_3^- secretion (Chang et al., 1983; Liu and Coupar, 1997). Moreover, clinical trials have demonstrated the potential interest of α_2-adrenoceptor agonists for the treatment of patients suffering cancer therapy-related diarrhoea or short bowel syndrome (Ippoliti, 1998; Schworer et al., 1995). It is thus possible that the decrease of epithelial α_2-adrenoceptor may contribute to harmful cytokine effects observed in inflammatory intestinal diseases. Moreover, numerous molecules inhibiting cytokine action have been developed and tested in clinical trials. An antibody against IFNγ (fontolizumab) did not demonstrate efficacy when administered at a single dose in patients with Crohn's disease, but was more efficient when a second dose was administered later (Nakamura et al., 2006). By contrast, anti-TNFα agents such as infliximab, CDP571 or CDP870, which are mouse/human chimeric antibodies, gave very promising results in the treatment of Crohn's disease, although adverse effects are also described (Nakamura et al., 2006; Sandborn and Faubion, 2004). It is possible that maintenance of α_2-adrenoceptor expression, which may occur following the treatment with anti-TNFα antibodies, would represent a part of beneficial effects of these molecules.

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