Potentiation of cytokine induced iNOS expression in the human intestinal epithelial cell line, DLD-1, by cyclic AMP

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Abstract

Background—Nitric oxide production by the inducible isoform of nitric oxide synthase (iNOS) is thought to play a role in the pathogenesis of inflammatory bowel disease along with other proinflammatory mediators.

Aims—To examine the effects of cAMP, an intracellular mediator of several proinflammatory mediators, on iNOS expression in the human intestinal epithelial cell line, DLD-1.

Methods—iNOS activity was assessed by measuring the NO stable oxidative product NO₂⁻. iNOS protein expression and iNOS mRNA levels were determined by western blotting and northern blotting, respectively.

Results—iNOS activity, protein, and mRNA were induced by a combination of interleukin 1β (0.5–5 ng/ml), interferon γ (20–200 u/ml), and tumour necrosis factor α (10–100 ng/ml). The cytokine induced NO2 activity was potentiated by N'6,2'6-O-dibutyryladenosine 3':5'-cyclic monophosphate and 8-bromoadenosine 3':5'-cyclic monophosphate (0.1–1 mM), and the adenylyl cyclase activator, forskolin (1–100 µM). This activity was inhibited by the selective iNOS inhibitor, 1400W (0.1–100 µM). These agents increased iNOS protein. The cAMP analogues potentiated iNOS at the transcriptional level as shown by effects of actinomycin D (5 µg/ml) and northern blot analyses; the nuclear factor (NF) κB inhibitor, pyrrolidine dithiocarbamate (10–200 µM), significantly reduced this potentiation. The cAMP potentiated iNOS activity was inhibited by the tyrosine kinase inhibitor, A25 (10–200 µM) and the Janus activated kinase 2 inhibitor, B42 (10–200 µM).

Conclusions—Increased intracellular cAMP is a potent stimulus of iNOS expression in combination with cytokines in DLD-1 cells, acting at the transcriptional level and involving NF-κB and the JAK-STAT pathways. Thus, proinflammatory mediators that increase cAMP levels may augment iNOS expression and NO production.

Keywords: inducible nitric oxide synthase colonic epithelial cells; nuclear factor κB; cytokines; cyclic AMP; JAK-2

The inducible isoform of nitric oxide synthase (iNOS) (EC 1.14.13.39) is capable of sustained production of high quantities of nitric oxide (NO), which is thought to be cytotoxic and to mediate deleterious effects. The iNOS isoform is expressed in a wide range of cells, mainly following exposure to cytokines and lipopolysaccharides.

The expression of iNOS and the NO produced or its subsequent products could play a key role in the pathogenesis of inflammatory bowel disease (IBD). Indeed, overexpression of iNOS protein, increased iNOS activity or NO release, and increased iNOS mRNA level, both in ulcerative colitis and in Crohn’s disease, have been shown. Furthermore, besides macrophages and polymorphonuclear cells infiltrating mucosa and submucosa, intestinal and colonic epithelial cells represent a major source of NO during inflammatory processes including IBD.

The nitric oxide pathway is known to interact with other systems, which are likely to be involved in the pathogenesis of IBD such as cyclooxygenase 2 (COX-2) and the adenosine 3':5'-cyclic monophosphate (cAMP) pathways. COX-2 mRNA levels are increased in acute IBD and COX-2 protein is overexpressed along with iNOS in the colonic and intestinal epithelial cells of patients with IBD. Furthermore, the products of COX-2 enzyme activity, such as prostaglandin E₂, have also been reported to be augmented in IBD. Cyclic AMP is thought to be the main intracellular mediator of proinflammatory prostaglandins through adenylyl cyclase activation and could also mediate some effects of proinflammatory cytokines such as interferon γ (IFN-γ) and interleukin 1β (IL-1β) in macrophages and vascular smooth muscle cells. It is noteworthy that increased colonic adenylate cyclase activity has been shown in tissue from ulcerative colitis.
tion, elevation of cAMP is thought to be responsible for secretory effects resulting in the diarrhoea observed in infectious enterocolitis and possibly in IBD.

In many cell types, the induction of iNOS can be modulated by cAMP. Indeed, cAMP has been shown to induce iNOS either alone or in combination with cytokines in mesangial cells, fibroblasts, vascular smooth muscular cells, macrophages, and cardiac myocytes. However, other reports suggest that cAMP may also act as an inhibitor of iNOS induction in these same cell types, including macrophages and mesangial cells, or in insulinoma cells, hepatocytes, Kupffer cells, or glial cells.

In human DLD-1 intestinal epithelial cells, iNOS can be induced by a combination of cytokines. This expression is controlled mainly at the transcriptional level and involves a tyrosine protein kinase pathway and the pleiotropic transcription factor, nuclear factor-kB (NF-kB). In the present study, the effects of cAMP on iNOS expression in the human epithelial intestinal cell line, DLD-1, have been investigated.

Material and methods

CELL CULTURE

The DLD-1 cell line was obtained from the European Collection of Cell Culture (Salisbury, UK) (No. 90102540) and used between passages 25 and 50. Cells were grown in Dulbecco modified Eagle's medium (DMEM) with 4 mM l-glutamine and 10% heat inactivated fetal calf serum. Cells were cultured at 37°C in a water saturated atmosphere of 95% air and 5% CO₂, refed every two days and passed weekly. Cells were allowed to grow for 72–96 hours to confluence before use. Cytokines and other agents were added to fresh medium without serum. Cytokines used for induction were IFN-γ, IL-1β, and tumour necrosis factor α (TNF-α).

CELL VIABILITY MEASUREMENT

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan. Cells grown in 96 well plates were incubated at 37°C for one hour with 0.4 mM MTT. Cells were solubilised in 100 µl dimethyl sulfoxide and absorbance was read at λ = 550 nm. Results were expressed as percentage of control (non-treated cells).

CELL COUNTING AND PROTEIN CONCENTRATION

Cells were dissociated in a solution of 0.25% trypsin and 3 mM EDTA in phosphate buffered saline (PBS) pH 7.4 (without calcium and magnesium). After five minutes cells were counted with a haemocytometer, adding trypan blue. Only cells that excluded dye were counted as viable cells. Results were expressed as number of viable cells per ml. The protein concentration was determined using a modification of the Bradford method (BioRad kit) and bovine serum albumin as a standard. Results were expressed as µg of protein per ml.

ASSESSMENT OF NOS ACTIVITY

Cells were stimulated in 96 well plates as indicated above. After 24 hours' exposure, cell culture medium was used to determine nitrite/nitrate production as an index of NOS activity. To reduce nitrate (NO₃⁻), 50 µl of medium was transferred in a 96 well plate and incubated for 15 minutes at 37°C with flavin adenine dinucleotide (50 µM), β-nicotinamide adenine dinucleotide phosphate, reduced form (500 µM), and nitrate reductase from Aspergillus species (1 U/ml), and then five minutes further with lactate dehydrogenase (100 U/ml) and sodium pyruvate (100 mM). Then, 50 µl of Griess reagent (0.25 M phosphoric acid, 30 mM sulphanilamide, 2 mM naphthylethylene diamine) was added to each well. The resultant colour change was quantified by spectrophotometry (λ = 550–650 nm). Nitrate levels were determined using a sodium nitrate standard curve and expressed as µM/10⁶ cells according to the cell number in each well.

WESERN BLOTTING

Cells were washed with ice cold PBS (pH 7.4) and homogenised in Tris-mannitol buffer (2 mM Tris 7–9, 50 mM mannitol, 100 mM phenyl methyl sulphonl fluoride, 2 µM leupeptin, 0.5 µM aprotinin, 0.5% Triton X-100), using a glass/glass homogeniser. Homogenates were sonicated twice for 10 seconds on ice and spun for 15 minutes at 21,000 g at 4°C. Aliquots of 100 µg of total cellular protein were denatured by mixing and boiling with 20 mM Tris 7–9, 2 mM EDTA, 2% sodium dodecyl sulphate (SDS), 10% β mercaptoethanol, 20% glycerol. The samples were electrophoresed on 7.5% SDS-polyacrylamide gel, and then transferred to nitrocellulose membrane (Amersham, Little Chalfont, UK). After blocking with PBS (pH 7.4), 0.25% Tween 20 (v/v), and 5% non-fat dried milk, membranes were probed with anti-iNOS monoclonal antibody at a 1/2000 dilution (Transduction Laboratories, Lexington, UK) for one hour at room temperature, washed with PBS-Tween 20, and then incubated with horseradish peroxidase conjugated second antibody (1/4000 dilution) for one hour at room temperature. Membranes were developed, using an enhanced chemiluminescence system (Amersham) and exposed to Hyperfilm (Amersham). Films were analysed using the Molecular Analyst Software (BioRad Laboratories, Hercules, USA) after scanning on a densitometer (GS-700 Imaging Densitometer, BioRad Laboratories).

NORTHERN BLOTTING

The iNOS cDNA probe was obtained by polymerase chain reaction (PCR) amplification of iNOS RNA from cytokine induced DLD-1 cells. The following primers were used to amplify the 3590–3848 bp region of human iNOS cDNA according to the published sequence (GenBank accession number: L09210), leading to a 259 bp fragment: 5'-CGG TGC TGT ATT TCC GTA CGA GGC
GAA GAC GAC and 5'-GCT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC. Then, the positive band was excised from the agarose gel and cDNA purified using a commercially available kit (Geneclean kit, Bio 101 Inc., La Jolla, California, USA).

Total RNA from cell monolayers was extracted using Trizol (Gibco BRL, Paisley, Scotland, UK). The amount of RNA was calculated from optical density measurements at \( \lambda = 260 \) nm. A 10 \( \mu \)g aliquot of total RNA was loaded on a 1% denaturing agarose gel, containing 2 M formaldehyde and 6 mM 3(N-morpholino)propanesulphonic acid. RNA was transferred onto an uncharged nylon membrane followed by hybridisation (Stratagene QuickHyb hybridisation solution, Cambridge, UK). The iNOS cDNA was radiolabelled with \( ^{32}P \)-dCTP by the random primer method (Multiprime DNA labelling system, Amersham). A photograph of agarose gel stained with ethidium bromide was taken as control of equivalent loading between lanes. Films were analysed as described for western blots.

**STATISTICAL ANALYSIS**

Data are shown as mean (SEM) of at least three independent experiments, each done in triplicate. Northern and western blots are shown as a representative photograph of three independent experiments. Statistical significance was assessed by Student’s \( t \) test where \( p<0.05 \) was taken as significant.

**CHEMICALS**

The iNOS antibody was from Transduction Laboratories (Afinity Research Products, Exeter, UK). Human TNF-\( \alpha \) was from R&D Systems (Abingdon, UK). Nitrate reductase was from Boehringer Mannheim (Lewes, UK). Methanol and ethanol were from BDH Laboratories Supplies (Lutterworth, UK). Bis-acrylamide solution and protein assay kit were from BioRad Laboratories (Hertfordshire, UK). DMEM, non-essential amino acids, Trizol, and primers were obtained from Gibco BRL (Paisley, UK). Tyrphostins A25 (\( \alpha \)-cyano-(3,4,5-trihydroxy)cinnamonic acid) and B24 (N-benzyl-3,4-dihydroxybenzylidenecyanamide) were from Calbiochem (Nottingham, UK) and were prepared in dimethylsulphoxide, the final concentration of which did not exceed 0.2%. Pyrrolidine dithiocarbamate (PDTC; Sigma, Poole, UK) was dissolved in phosphate buffered saline (PBS; pH 7.4). 1400W (N-(3-(aminomethyl)benzyl)acetamide) was a kind gift from Dr R Knowles (GlaxoWellcome Research, Stevenage, UK). All other compounds and chemicals were purchased from Sigma (Poole, UK).

**Results**

**CYCLIC AMP EFFECTS ON NOS ACTIVITY**

In DLD-1 cells, incubation with two cAMP analogues, \( N',2'-O \)-dibutyryladenosine 3':5'-cyclic monophosphate (Db-cAMP; 0.1–1 mM) or 8-bromoadenosine 3':5'-cyclic monophosphate (8Br-cAMP; 0.1–1 mM) alone caused no increase in \( NO_2^- \) production compared with control cells. Thus, \( NO_2^- \) levels were 0.8 (0.3) µM/10⁶ cells. Results are expressed as percentage of induction with cytomix alone and represent means (SEM) from at least three different experiments, each done in triplicate. **\( p<0.01 \), ***\( p<0.001 \) compared with value in the cells treated by cytomix alone. \( \dagger\)\( p<0.001 \) compared with value in the cells treated with vehicle alone or cytomix and coincubated with 2’-O-methyl adenosine (MA), in DLD-1 cells. Cells were treated with vehicle alone or cytomix and coincubated with 8Br-cAMP alone or MA. Results are expressed as means (SEM) from at least three different experiments, each done in triplicate. **\( p<0.01 \) compared with value in the cells treated by cytomix alone; \( \dagger\)\( p<0.001 \) compared with value obtained in the cells treated by cytomix and 8Br-cAMP.

![Figure 2](http://gut.bmj.com/content/45/3/367/F2)

**Figure 2** Potentiation of cytomix induced iNOS activity by 8Br-cAMP was reversed by the cAMP antagonist, 2’-O-methyl adenosine (MA), in DLD-1 cells. Cells were treated with vehicle alone or cytomix and coincubated with 8Br-cAMP alone or MA. Results are expressed as means (SEM) from at least three different experiments, each done in triplicate. **\( p<0.01 \) compared with value in the cells treated by cytomix alone; \( \dagger\)\( p<0.001 \) compared with value obtained in the cells treated by cytomix and 8Br-cAMP.

![Figure 3](http://gut.bmj.com/content/45/3/367/F3)

**Figure 3** Potentiation of cytomix induced iNOS activity by forskolin (FSK) and Db-cAMP in DLD-1 cells. Cells were incubated in serum free medium with cytomix alone (white bars) or FSK, with or without 3-isobutyl-1-methylxanthine (IBMX) or Db-cAMP. Results are expressed as percentage of induction with cytomix alone and represent means (SEM) from at least three different experiments, each done in triplicate. **\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \) compared with value obtained in cytomix treated cells.
µM/10⁶ cells, 1.7 (0.9) µM/10⁶ cells, and 0.85 (0.4) µM/10⁶ cells in control cells, Db-cAMP (1 mM) treated cells, and 8Br-cAMP (1 mM) treated cells, respectively (p>0.05).

A combination of IFN-γ (200 u/ml) and IL-1β (5 ng/ml) for 24 hours increased NO₂⁻ levels to 41.4 (3.1) µM/10⁶ cells. Coincubation with Db-cAMP (0.1–1 mM) and 8Br-cAMP (0.1–1 mM) led to a dose dependent potentiation of the NO₂⁻ production induced by the combination of these two cytokines (fig 1).

8Br-cAMP and Db-cAMP also increased the NO₂⁻ production induced by a combination of IL-1β, IFN-γ, and TNF-α at low concentrations (0.5 ng/ml, 20 u/ml, and 10 ng/ml, respectively). The latter mixture led to an NO₂⁻ production of 36.3 (8.3) µM/10⁶ cells, which was significantly increased to 106.2 (26.7) µM/10⁶ cells and 170.5 (3.7) µM/10⁶ cells with 1 mM 8Br-cAMP and 1 mM Db-cAMP, respectively (p<0.05 and p<0.001, respectively).

8Br-cAMP and Db-cAMP also increased significantly the NO₂⁻ production provoked by incubation with the combination of the three cytokines for 24 hours at the higher concentrations, IFN-γ (200 u/ml), IL-1β (5 ng/ml), and TNF-α (100 ng/ml) (cytomix) as shown in figs 2 and 3.

The guanosine 3′:5′-cyclic monophosphate analogue, 8Br-cGMP (0.1–1 mM) had no effect on NO₂⁻ production either when used alone or in combination with any cytokine mixture (100.7 (3.7)% and 96.3 (8)% of the cytokine induced NO₂⁻ production with 8Br-cAMP 0.1 and 1 mM, respectively).

An activator of adenylate cyclase, forskolin (1–100 µM), also significantly potentiated cytokine induced NO₂⁻ production with a maximal effect at 10 µM forskolin (fig 3). No further increase was observed at higher concentrations. No effect was observed with
forskolin in the absence of cytokine (1.2 (0.3) µM/10^6 cells with forskolin 10 µM; p>0.05 compared with control cells). The potentiation of cytokym-induced NO_2^- production by forskolin was further significantly augmented by coincubation with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX; 100 µM) as shown in fig 3. IBMX did not increase NO_2^- production when added to cytokym alone, being 121 (3)% the NO_2^- release provoked by cytokym alone.

Incubation with the cAMP antagonist, 2'-O-methyl adenosine (5 mM), significantly reversed the 8Br-cAMP and Db-cAMP potentiation of cytokym induced NO_2^- production (fig 2). In addition, 2'-O-methyl adenosine (5 mM) was also able to decrease by 35.5 (3.4)% the NO_2^- production observed in cells treated with 8Br-cAMP alone (p<0.01).

The cAMP potentiated NO_2^- production was confirmed to be the result of iNOS expression, by its concentration dependent inhibition by the selective iNOS inhibitor, 1400W, IC_50 of 7.15 (0.8) µM. Furthermore, western blot analyses using a monoclonal iNOS antibody showed increased iNOS protein expression in DLD-1 cells treated with 8Br-cAMP (1 mM) and cytokym compared with cells treated with cytokym alone (fig 4A).

\[\text{EFFECTS OF TYRPHOSTINS A25 AND B42 ON cAMP POTENTIATED iNOS ACTIVITY}\]

The tyrphostins A25 (10–200 µM) and B42 (10–200 µM) were prepared in dimethylsulphoxide (DMSO). The maximal final concentration of DMSO was 0.2% and this concentration had no significant effect on cytokym induced NO_2^- production (96.1 (5.1)% of control, n=3). The tyrphostins A25 and B42 did not significantly affect cytokym inducing NO_2^- production by 8Br-cAMP and cytokymes was decreased by 18.5 (3.5)% with PDTC (200 µM) as shown in fig 4B. Cycloheximide (5 µg/ml) also substantially reduced this iNOS mRNA level as shown (fig 4B).

\[\text{CYCLIC AMP EFFECTS ON iNOS mRNA}\]

The iNOS mRNA was detected at six hours by northern blot analysis in cytokym treated DLD-1 cells but not in control vehicle treated cells. Following coincubation of the cells with cytokym and 8Br-cAMP (1 mM), a further increase was observed varying from 140% to 170% by densitometric analyses (range from three experiments) compared with the level in the cytokym treated cells. Actinomycin D (5 µg/ml) abolished this signal (fig 4B). Treatment with actinomycin D (5 µg/ml) also decreased the cAMP potentiated NO_2^- production, to 1.4 (0.8) µM/10^6 cells, reaching the basal level.

\[\text{Results expressed as mean (SEM) from at least three different experiments. The number of viable cells was assessed by the trypan blue exclusion test. Statistical significance was assessed by Student's t-test. **p<0.01, ***p<0.001 compared with control.}\]

**Table 1**  Effect of cAMP analogues on cell viability in DLD-1 cells exposed for 24 hours to cytokym (CM)

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Viable cells (10^6/ml)</th>
<th>MTT assay (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.254 (0.023)</td>
<td>100</td>
</tr>
<tr>
<td>CM</td>
<td>0.246 (0.033)</td>
<td>104.5 (2.7)</td>
</tr>
<tr>
<td>CM + 8Br-cAMP 0.1 mM</td>
<td>0.203 (0.022)**</td>
<td>86.6 (4)**</td>
</tr>
<tr>
<td>CM + 8Br-cAMP 1 mM</td>
<td>0.151 (0.009)**</td>
<td>74.8 (4.8)**</td>
</tr>
<tr>
<td>CM + Db-cAMP 0.1 mM</td>
<td>0.213 (0.005)**</td>
<td>90.5 (5.1)**</td>
</tr>
<tr>
<td>CM + Db-cAMP 1 mM</td>
<td>0.185 (0.012)**</td>
<td>85.2 (6.0)**</td>
</tr>
</tbody>
</table>

Cytokym + 8Br-cAMP (1 mM)

\[\text{CM (PBS)}\] had no effect on cytokym induced NO_2^- production. The level of iNOS mRNA induced by 8Br-cAMP and cytokymes was decreased by 18.5 (3.5)% with PDTC (200 µM) as shown in fig 4B.

\[\text{CYCLIC AMP AND CELL VIABILITY}\]

Dose dependent cytotoxicity was observed in cells treated with cytokym and 8Br-cAMP or Db-cAMP, as estimated by the MTT assay and the trypan blue exclusion (table 1). Neither 1400W (100 µM) nor 2'-O-methyl adenosine (5 mM) reversed this toxicity (table 2). Similar cytotoxicity was observed in cells treated with cAMP analogues alone. Forskolin was also cytotoxic to DLD-1, but only at the highest concentration, with a cell viability of 95 (2)%
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Results expressed as mean (SEM) from at least three di

cytokine exposure.34 In contrast, recent re-

tests could reflect the cell and species

details in cyclic

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Discussion

In this study, using cAMP analogues, as well as the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, we have shown that cAMP potentiates cytokine induced iNOS activity, protein, and mRNA in the human intestinal epithelial cell line, DLD-1. Cyclic AMP has been shown to induce iNOS expression in many cell types but the majority of these are rat cell lines. Indeed, cAMP responsive element (CRE) sites have been identified in the rat iNOS gene39 and could be involved in the iNOS expression caused by cAMP alone or in combination with cytokines. Nevertheless, cAMP can also decrease iNOS expression in cells of rat origin.28 32 40 To our knowledge, only two studies have reported cAMP enhancement of iNOS expression in human cells, these being monocytes and T cells.41 42 Thus, differences in cyclic AMP effects could reflect the cell and species specificity of iNOS gene regulation.

As shown by northern blot analyses and the effects of the transcription inhibitor, actinomycin D on iNOS activity and mRNA expression, this potentiation is mainly controlled at the transcriptional level. Increased intracellular concentrations of cAMP stimulate several pathways, including the protein kinase A (PKA) pathway. Within the nucleus, PKA phosphorlates the cAMP responsive element binding protein (CREB), enhancing its DNA binding activity.43 As there is no reported CRE binding sequence in the 5' flanking region of the iNOS gene in DLD-1 cells,7 30 cAMP potentiation of iNOS expression is unlikely to reflect a direct effect on the iNOS gene through the CREB/ATF (CREB/activating transcription factor) family of transcription factors. Nevertheless, cAMP and PKA pathways have been shown to be involved in the activation of some other transcription factors which are thought to play an important role in iNOS gene activation in DLD-1. Indeed, cAMP may induce iNOS gene transcription through activation of NF-κB directly43 or through PKA dependent phosphorylation of the IκB subunit.44 45 Cyclic AMP, through activation of CREB, may also compete directly with AP-1,46 which has been recently reported to be a nega-

tive regulator of iNOS in DLD-1 cells.36

Recently, forskolin (100 µM) and Db-cAMP (100 µM), either alone or in combination with cytokine, have been reported to produce no increase in the iNOS mRNA level in DLD-1 cells.35 However, in the present study, forskolin was active at a concentration of 100 µM but was less potent than at a concentration of 1 µM and 10 µM. These effects of forskolin were further augmented in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine. Db-cAMP, which is considered resistant to phosphodiesterase activity, was only slightly effective at 100 µM in our studies, the maximum potentiation being obtained with 1 mM of the dibutyl and 8Br-cAMP analogues. Others have also shown that only high concentrations of Db-cAMP (0.1–1 mM) are able to increase iNOS in rat mesangial cells.53 Nevertheless, the similar effect that we observed with two lipophilic cAMP analogues, as well as an adenylate cyclase activator, but not with a corresponding analogue of cyclic GMP, at the level of the iNOS mRNA, protein and activity suggest that cAMP has a role in iNOS modulation in DLD-1 cells.

The effect of the putative NF-κB inhibitor, PDTC, on NO2− production and iNOS mRNA levels suggests that cAMP potentiated iNOS transcription involves this transcription factor. These results are in agreement with previous reports of iNOS upregulation in DLD-1 after cytokine exposure.14 In contrast, recent reports, detecting mRNA only, suggest that this pathway is not a major effector of iNOS gene transcription in this cell type.36 In the present study, we could obtain only about 50% inhibition of iNOS activity and the level of the iNOS mRNA was hardly decreased with high concentrations of PDTC (200 µM). However, the translation inhibitor, cycloheximide was able to reduce substantially the iNOS mRNA level after exposure to cAMP/cytokine. These findings support the suggestion that some other transcription factors could participate in iNOS gene transcription and/or that post-transcriptional events, such as mRNA stabilisation, could occur in regulating iNOS expression.34 36 38

The IFN-γ activated kinase JAK-2 is known to tyrosine phosphorylate STAT1α, which is subsequently translocated into the nucleus to bind specific DNA sites.9 It has been recently proposed that the JAK-STAT pathway could play an important role in iNOS induction in DLD-1 cells.9 Our results confirm these findings, showing that the iNOS activity induced by cAMP/cytokine incubation was inhibited by the tyrosine kinase inhibitor A25 and by the specific JAK-2 inhibitor, B42.50 As abolition of iNOS activity was observed with B42, JAK-2 activation is strongly implicated in iNOS induction in DLD-1 cells. Nevertheless, our findings suggest that the STAT and NF-κB pathways are both involved in the cAMP/cytokine iNOS induction in DLD-1 cells.

The potentiation of cytokine induced iNOS activity was inhibited by the cAMP antagonist, 2'-O-methyl adenosine, suggesting specific interactions with cAMP receptors. Furthermore, the iNOS activity provoked by cytomix alone was also partially inhibited by 2'-O-methyl adenosine. This result suggests that induction of

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<tr>
<td>CM + 8Br-cAMP 1 mM</td>
<td>74.8 (4.8)</td>
</tr>
<tr>
<td>CM + 8Br-cAMP 1 mM + MA (5 mM)</td>
<td>77.5 (7.5)</td>
</tr>
<tr>
<td>CM + 8Br-cAMP 1 mM + 1400W (100 µM)</td>
<td>77.4 (8.6)</td>
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Results expressed as mean (SEM) from at least three different experiments. There was no statistically significant difference (Student's t test) between the three treatments.
iNOS by cytokines is partly modulated by the endogenous cAMP in DLD-1 cells, as reported in non-intestinal cell types with IL-1β or IFN-γ. 17,18

In the present work, the cAMP analogues were cytotoxic to DLD-1 cells following 24 hours incubation. NO production or iNOS activation are unlikely to be involved as the cAMP analogues were equally toxic with or without cytokine. Furthermore, the selective iNOS inhibitor, 1400 W did not attenuate this cytotoxicity in concentrations that inhibited the iNOS activity induced by cytokym. The decrease in cell viability is unlikely to be directly related to the intracellular increase in cAMP concentration, as the cAMP antagonist, 2'-O-methyl adenosine did not modify this cytotoxicity and the mechanism of this action is therefore unknown.

These results emphasise the possible relation between the iNOS pathway and other proinflammatory mediators that act through cAMP mediated pathways. Such findings support the concept that cAMP may act as an important intracellular mediator in inflammation, being additionally involved in the regulation of iNOS expression provoked by cytokines in intestinal or colonic epithelial cells.

The authors are indebted to Ms Elizabeth Wood for supplying the cultured cells. They would like to acknowledge Dr Linda Gibbs for her help in performing northern blot analyses. MG is a recipient of a grant from Institut de Recherche pour les Maladies du Sang to which she is also grateful. The authors are indebted to Ms Elizabeth Wood for supplying the cultured cells. They would like to acknowledge Dr Linda Gibbs for her help in performing northern blot analyses. MG is a recipient of a grant from Institut de Recherche pour les Maladies du Sang to which she is also grateful.


