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Effects of nitric oxide on proliferation and differentiation of rat brown adipocytes in primary cultures

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1 In the present work, we study the effect of NO on the proliferation and differentiation of brown fat cells in primary cultures.

2 Brown fat precursor cells isolated from rat brown adipose tissue were cultured for 8 days until confluence and treated daily with the NO donating agents, S-nitroso-acetyl penicillamine (SNAP) or S-nitroso-L-glutathione (GSNO). Both agents (300 μM) decreased cell proliferation approximately 8 fold on day 8. The inhibitory effect of NO was unlikely to be due to cytotoxicity since (i) cells never completely lost their proliferation capacity even after 8 days of exposure to repeated additions of SNAP or GSNO, and (ii) the inhibitory effect was reversible after removal of the media containing NO donors.

3 Daily treatment with nitric oxide synthase inhibitors, such as N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM), led to the stimulation of cell proliferation by $44 \pm 5\%$, $n = 3$, suggesting that NO, endogenously produced in brown adipocytes, may be involved in modulating cell growth.

4 Daily treatment with both SNAP or GSNO induced significant mitochondriogenesis, measured as the mitochondrial conversion of 3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyl tetrazolium bromide (MTT) to formazan, whilst daily treatment with L-NAME was without effect.

5 The inhibition of cell proliferation by NO donors was accompanied by the expression of two genes coding for peroxisome proliferator activated receptor-γ and uncoupling protein-1, which are upregulated during differentiation.

6 Increasing cyclic GMP in cells by 8-bromo-cyclic GMP (100–1000 μM) did not reproduce the observed NO effects on either cell number or gene expression. On the other hand, chronic treatment with the inhibitor of the NO-stimulated guanylyl cyclase, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), reduced the expression of peroxisome proliferator activated receptor-γ and uncoupling protein-1.

Keywords: Brown adipocytes; nitric oxide; uncoupling protein-1; peroxisome proliferator-activated receptor-γ; cell proliferation; cell differentiation; energy expenditure; obesity

Introduction

Brown adipose tissue (BAT) is an important site of energy expenditure (Trayhurn & Nicholls, 1986), that is functionally quiescent and atrophied in obesity (Himms-Hagen & Desautels, 1978). Exposure to cold induces the recruitment process (Cannon & Nedergaard, 1996; Cannon *et al*., 1996), which consists of a series of events that transiently transform a proliferatively dormant into a hyperplastic organ in which cell proliferation and differentiation are accompanied by the multiplication and functional activation of mitochondria that is an index of the differentiation process (Cannon & Nedergaard, 1996). The precise molecular mechanisms that are involved in proliferation and differentiation of brown fat cells are not known at present. Whilst *in vitro* and *in vivo* evidence indicates that noradrenaline (NA) plays an important role in all of these events (Cannon & Nedergaard, 1996), experiments in cell cultures derived from young rat brown fat precursor cells indicate that cells can proliferate to confluence and initiate the differentiation process characteristic of brown adipocytes in the total absence of NA, the role of which may be that of reinforcing rather than triggering the differentiation process (Néchad *et al*., 1987).

We have recently reported that rat brown adipocytes differentiated in culture express an inducible nitric oxide synthase (iNOS) isoform, similar to the iNOS of macrophages, and thus synthesize and release nitric oxide (NO) (Nisoli *et al*., 1997). One of the many physiological functions of NO (reviewed in Bredt & Snyder, 1994; Garthwaite & Boulton, 1995) is to control proliferation and differentiation in various cell systems (Garg & Hassid, 1989; Lepoivre *et al*., 1990).

The purpose of the present work was to determine the effects of NO on the proliferation and differentiation of rat brown adipocytes. Our results show that NO is capable of (i) inhibiting proliferation, an event which seems to be essential for the differentiation of proliferating brown adipocytes, and (ii) triggering the differentiation programme by modulating the expression of peroxisome proliferator activated receptor-γ (PPARγ), a gene known to play a priming role in adipose tissues (Tontonoz *et al*., 1994a,b; Sears *et al*., 1996). These functions are variously independent of or dependent on cyclic GMP generation. On the basis of these data, we conclude that NO may play a significant role in proliferation/differentiation programme of brown adipocytes.

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Methods

Brown adipocyte isolation

Brown fat precursor cells were isolated from the interscapular brown fat of male Sprague-Dawley rats (150–160 g body weight) (Harlan Nossan, Correzzana, Italy), which were kept in standard laboratory conditions (12 h light/dark cycle; food and water *ad libitum*) as previously described (Néchad *et al*., 1983; Nisoli *et al*., 1996). All of the animal experiments were carried out in accordance with the highest standards of humane animal care. The interscapular brown adipose tissue (BAT) fragments (12–15% w v^{-1}) were carefully dissected out under sterile conditions, and then placed in HEPES-buffered solution (consisting of 123 mM Na^+, 5 mM K^+, 1.3 mM Ca^{2+}, 131 mM Cl^-, 5 mM glucose, 1.5% w v^{-1} crude bovine serum albumin and 100 mM HEPES, pH-adjusted with NaOH to 7.4) described by Néchad *et al*. (1983) for the isolation of the brown fat precursor cells, supplemented with 0.2% w v^{-1} type II collagenase. After 30 min at 37°C, the tissue remnants were removed by filtration through a 250 μm nylon screen, and the mature adipocytes were allowed to float to the surface (30 min on ice). The infranatant containing the adipocyte precursor cells was then collected with a 2 mm metal syringe (mature cells and lipid layer discarded), filtered through a 25 μm nylon screen, pelleted by centrifugation for 10 min at $700 \times g$ in 10 ml of culture medium and diluted in 20 ml of the same medium.

Adipose cell culture and treatment

Three million cells seeded in 24-well culture plates (Nunclon Delta) were cultured under a water-saturated atmosphere of 6% CO_2 in air at 37°C in 2.0 ml of a culture medium consisting of Dulbecco's modified Eagles's medium (DMEM) supplemented with 4 mM glutamine, 10% w v^{-1} newborn calf serum, 4 nM insulin, 4 nM triiodothyronine, 10 mM HEPES, 50 IU of penicillin, 50 μg ml^{-1} of streptomycin, and 25 μg ml^{-1} of sodium ascorbate (all from Flow Laboratories, Milan, Italy). Final concentrations of NO donors, GSNO and SNAP (100–1000 μM), NOS inhibitors, L-NAME or L-NIO (300 μM), or 8-bromo-cyclic GMP (100–1000 μM) were added to the cultures once a day from day 1 to day 8 (i.e. until the time of confluence and differentiation), when the medium was completely exchanged with fresh prewarmed medium. The cells were harvested 24 h after the addition of the agents for 8 days. The medium was discarded, the wells were washed twice with 2 ml of ice-cold phosphate-buffered saline (PBS) (Sigma, Milan, Italy) and the cells were scraped out with a disposable cell scraper (Nunc, Milan, Italy) for mRNA extraction for PCR analysis. For cell counting, 0.5 ml PBS containing 0.05% w v^{-1} trypsin and 0.02% w v^{-1} EDTA without calcium and magnesium was added to each well, and the cells were sedimented by 5 min of centrifugation at $700 \times g$. The sedimented cells were diluted in 0.5 ml culture medium and counted in a Bürker chamber.

MTT staining

For colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Denizot & Lang, 1986), the cells were seeded in 24-well plates (12.5×10^4 cells cm^2) and incubated in 0.4 ml of DMEM, supplemented as described above. They were incubated in medium with or without the compounds as indicated in the text. At the indicated time points, the cells were washed and 0.5 ml of filtered MTT stock solution (2.4 mM in RPMI 1640 wihout phenol red) were added, before the plates were incubated for 3 h at 37°C. At the end of this incubation period, the untransformed MTT was carefully removed and the dye crystals solubilized in 1 ml of 2-propanol. As a negative control, the absorbance of MTT was measured in cell-free plates treated in the same way. Absorbance was read immediately in a UVKON 941 spectrophotometer using a test wavelength of 570 nm and a reference wavelength of 690 nm.

PCR assay

Total cytoplasmic RNA was isolated from 1×10^6 cultured cells using the RNazol method (TM Cinna Scientific, Friendswood, Texas, U.S.A.). For PCR analysis, the RNAs were treated for 30 min at 37°C with 5 U ml^{-1} of RNase-free DNaseI in 100 mM Tris-HCl, pH 7.5 and 50 mM $MgCl_2$ in the presence of 2 U ml^{-1} of placenta RNase inhibitor. The concentration of RNA was determined by absorbance at 260 nm, and its integrity was confirmed by means of electrophoresis through 1% w v^{-1} agarose gels containing 0.1 mg ml^{-1} of ethidium bromide. One μg of total RNA was converted to cDNA using 200 U of Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, U.S.A.) in 20 μl of Promega-supplied buffer containing 0.4 mM dNTPs, 2 U ml^{-1} RNase inhibitor, 0.8 μg of oligo$(dT)_{12-18}$ primer (Sigma, Milan, Italy), and [^{32}P]dCTP. The cDNA was quantified by determining the amount of radioactivity incorporated into trichloroacetic acid-precipitable nucleic acid: usually, 100 ng of cDNA were synthesized from 1 μg of total RNA. A control experiment without reverse transcriptase was performed for each sample in order to verify that the amplification was not due to any residual genomic DNA. An aliquot (5% vol, ~20 ng) of the cDNA was amplified using the specific primers for UCP1 (reverse primer 5′-GTGAGTTCGACAACTTCCGAAGTG-3′; forward primer 5′-CATGAGGTCATATGTCACCAGCTC-3′) (Bouillaud *et al*., 1986), or PPARγ (reverse primer 5′-CATAAATAAGCTTCAATCGGATGG-3′; forward primer 5′-ATGCCATTCTGGCCCACCAACTTC-3′) (Zhu *et al*., 1993) with *Taq* DNA polymerase (Promega, Milan, Italy) in 25 μl of standard buffer (10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1% w v^{-1} Triton X-100, 2.5 mM $MgCl_2$, and 200 μM dNTPs). The PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and polymerization at 72°C for 45 s. After 35 cycles, a final 10-min incubation at 72°C was carried out. The mRNA for constitutive β-actin was separately examined as the reference cell transcript using specific primers as described by Gaudette & Crain (1991); the PCR conditions were: denaturation at 94°C for 30 s, annealing at 60°C for 20 s and polymerization at 72°C for 30 s. After 20 cycles, 5 μl of the PCR reaction mixtures obtained from the different treatment groups were added to 10 μl of the respective UCP1 or PPARγ PCR products. These reaction mixtures were separated by electrophoresis (2.0% w v^{-1} agarose gel in Tris-acetate-EDTA buffer, containing 0.1 mg ml^{-1} of ethidium bromide). The β-actin mRNA amplification products were equivalent in all of the cell lysates. The identity of the the PCR products was confirmed by hybridization using internal oligonucleotides obtained by means of the PCR amplification of cloned UCP1, PPARγ, and β-actin genes with the same specific primers as those described above (data not shown). The nitrocellulose

membranes were exposed for 8 h to NIF RX-100 films (Fuji).

Data analysis

The data are expressed as the means ± standard error of the mean (s.e.mean) of at least three independent experiments performed in triplicate. Comparisons were made using one-way analysis of variance followed by Student-Newman-Keuls *posthoc* comparisons, and P values of <0.05 versus control were considered significant.

Materials and drugs

The culture sera and media were purchased from GIBCO, Basel, Switzerland. The type II collagenase, fatty-acid free bovine serum albumin, and DNase I came from Boehringer Mannheim, Germany, and the S-nitroso-N-acetylpenicillamine (SNAP), propidium iodide, and S-nitroso-L-glutathione (GSNO) from Calbiochem, San Diego, CA, U.S.A. [1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one] (ODQ) was purchased from Alexis, Läufelfinger, Switzerland. 8-Bromo-cyclic GMP (8-bromo-cyclic GMP), and the remaining chemicals came from Sigma, Milan, Italy. Except for SNAP and ODQ (for which DMSO was used), the drugs were all dissolved in phosphate buffered saline (PBS). Appropriate controls solvents alone were used in parallel in all of the experiments; in the experiments involving L-N^Gnitro-arginine methyl ester (L-NAME), control experiments using the less active enantiomer, D-N^Gnitro-arginine methyl ester (D-NAME), led to results that were similar to those of the untreated controls. Oxyhaemoglobin (Hb) was prepared as reported by Feelish *et al.* (1996).

Results

Effects of prolonged exposure to NO donors on brown fat cell proliferation

Rat brown fat precursor cells, growing in culture and dividing rapidly, were used to study the effects of NO. After confluence (6–8 days), these mature, brown adipocyte-like cells start differentiating and finally acquire the ability to express the brown fat-specific uncoupling protein-1 (UCP1) (Nisoli *et al.*, 1996). Any relevant fibroblast contamination was excluded in this experimental system (Néchad *et al.*, 1983). In order to investigate the effect of NO on proliferation, the cultured brown fat cells were treated daily from day 1 to day 8 with or without different concentrations of GSNO or SNAP (ranging from 100–1000 μM), which liberate NO in solution (Morley *et al.*, 1993), and cells counted in a Bürker chamber. Neither compound affected the viability of the brown adipocytes when tested by trypan blue exclusion, and they were maximally effective in inhibiting cell proliferation at 300 μM. As shown in Figure 1A, GSNO and SNAP markedly inhibited the proliferation of cells: in control conditions, there are approximately eight times more cells on day 8 than after GSNO or SNAP treatment ($P<0.05$ for all treatment days). That cell proliferation was restored once the NO donors had been washed away confirms that this decrease in cell number was caused by decreased cell proliferation (cytostasis) and not cytotoxicity.

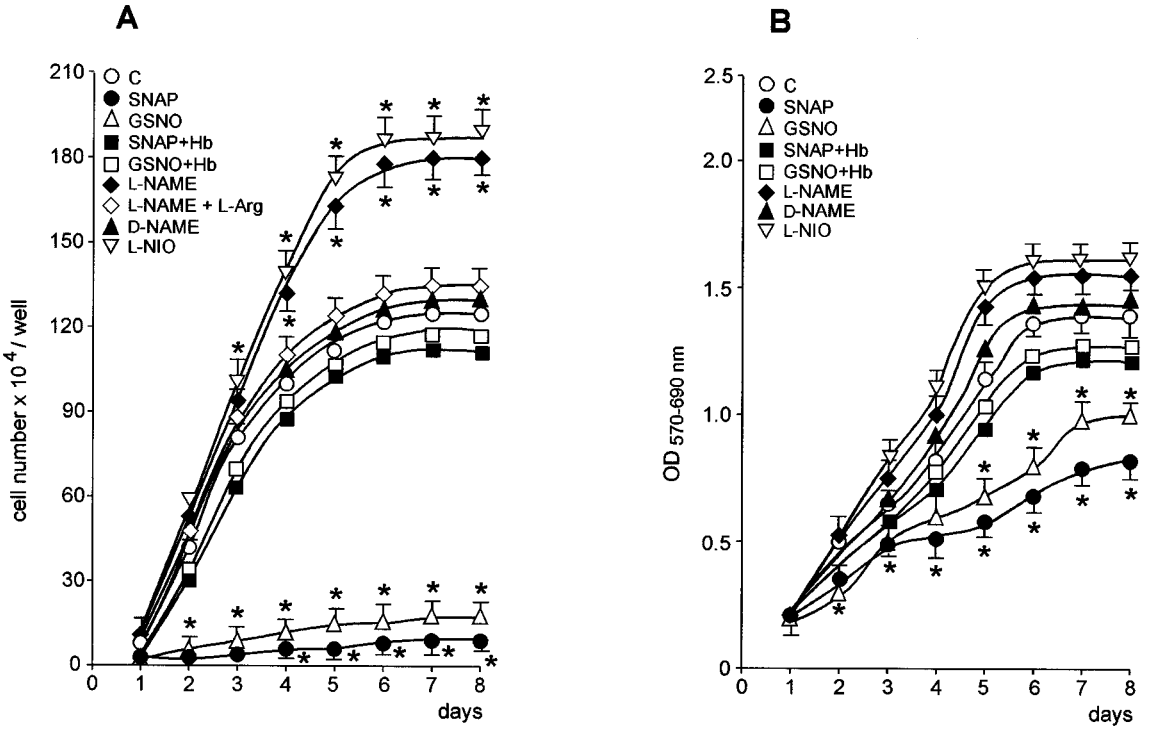

Figure 1 Effects of prolonged exposure to NO donors and L-NAME on cell number and the mitochondrial conversion of MTT to formazan in cultured brown fat cells. The cells were grown in culture medium without any further addition (C, controls) or with daily addition of 300 μM SNAP, GSNO, L-NAME, D-NAME, or L-NIO alone or in combination with 50 μM Hb or with 1 mM L-arginine. Hb and L-arginine did not have any effect *per se*. The cell number (A) and MTT metabolism (B) were determined. The experiments with Hb ($n=4$) were not performed in parallel. Each point represents the mean ± s.e.mean of four experiments performed in triplicate. *$P<0.001$ versus untreated cells.

To investigate whether the above inhibition by NO donors was indeed mediated through the generation of NO or due to additional actions of the compounds employed, cell proliferation experiments were carried out in the presence of oxyhaemoglobin (Hb), which binds and inactivates NO (Gross & Wolin, 1995). As shown in Figure 1A, SNAP and GSNO completely lost their inhibitory effect on cell proliferation when chronically administered in a medium supplemented with Hb (50 μM; see Sciorati *et al*., 1997).

Effect of prolonged exposure to NOS inhibitors on brown fat cell proliferation

If NO acts as an antiproliferative factor in growing brown fat cells, the inhibition of NOS should increase cell proliferation. Treatment with the NOS inhibitor L-NAME, at a concentration (300 μM) that is likely to inhibit all three enzyme isoforms (Knowles & Moncada, 1994), increased cell proliferation in comparison with controls (Figure 1A). Under the same experimental conditions, the less active enantiomer D-NAME (300 μM) did not alter cell proliferation when compared with controls (Figure 1A). This can be viewed as evidence that during unstimulated development in culture, endogenous NO exhibits an attenuating effect on proliferation. These findings were confirmed using other NOS enzyme inhibitors, such as L-*N*-(iminoethyl)-ornithine (L-NIO; 300 μM) (Figure 1A). In addition, the stimulatory effect of L-NAME was completely antagonized by 1 mM L-arginine (Figure 1A).

Effects of prolonged exposure to NO donors and NOS inhibitors on brown fat cell differentiation

The mitochondrial conversion of 3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyl tetrazolium bromide (MTT) to formazan, a method usually used for the measurement of both cell proliferation and cytotoxic effect on several types of cells (Mosmann, 1983), was taken as an index of mitochondriogenesis and, thus, of brown fat cell differentiation. Figure 1B shows that after chronic treatment with SNAP or GSNO (300 μM) the MTT signal was only reduced by 30–40% in spite of the very marked inhibition of cell proliferation (Figure 1A). This would indicate that each cell was more differentiated than in the controls. The presence of 50 μM Hb blocked this inhibition by the NO donors (Figure 1B). Interestingly, the active proliferative effect of L-NAME and L-NIO was not accompanied by a marked increase in MTT staining (Figure 1B), thus indicating that the differentiation mechanism was not enhanced in actively proliferating cells. D-NAME (300 μM) did not affect MTT staining (Figure 1B).

In order to investigate further the role of NO in the differentiation cascade, we examined whether the process can be modified by treatment with exogenous NO using two genes that are known to be upregulated at the very beginning (PPARγ) and as a consequence of brown fat cell differentiation (UCP1). Two PPARγ isoforms have recently been described: PPARγ1 (Zhu *et al*., 1993) and the adipose tissue-specific isoform PPARγ2, which differs from PPARγ1 by the presence of 30 additional amino acids at the N-terminal (Tontonoz *et al*., 1994a). The PPARγ primers used in our PCR experiments recognize both PPARγ1 and PPARγ2. UCP1 expression was analysed as a member of the growing UCP family, which now also includes the recently described UCP2 and UCP3 (Fleury *et al*., 1997; Boss *et al*., 1997). Semiquantitative PCR analyses using PPARγ and UCP 1 specific primers were made of the total RNA harvested daily from the cells (Figure 2), with the β-actin gene serving as an internal standard to allow between-sample comparisons. The signal intensity of each 35-cycle PCR product was quantified and normalized in relation to control

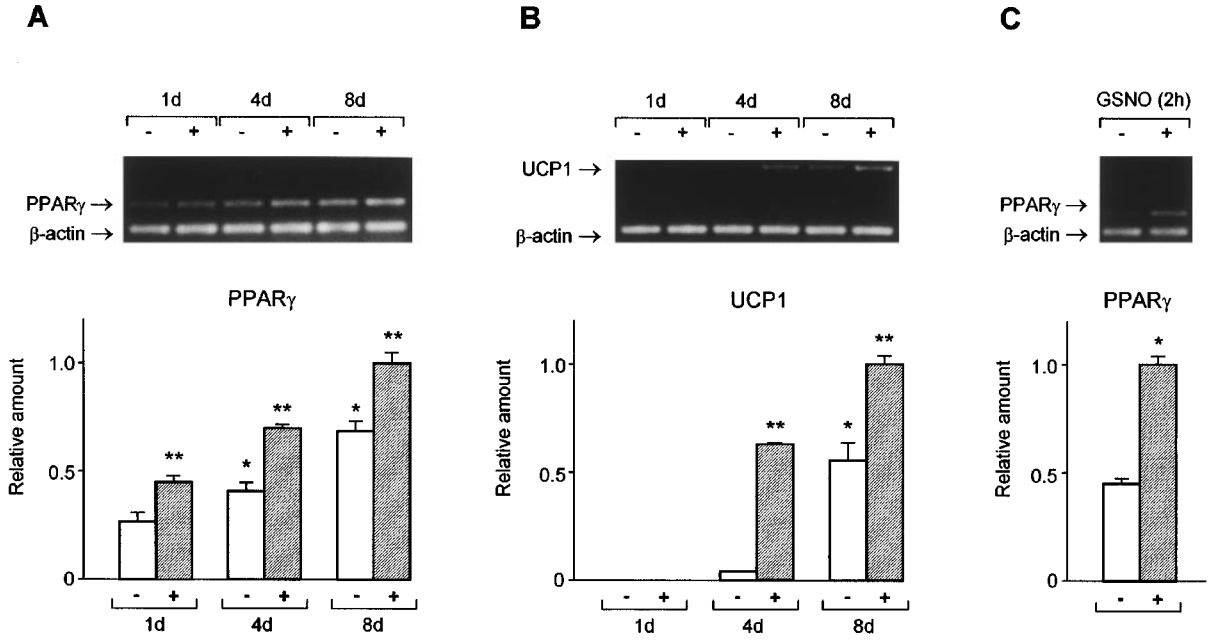

Figure 2 Effects of GSNO on PPARγ and UCP1 mRNA levels in cultured brown fat cells. The cells were chronically treated (+ and closed bars) with 300 μM GSNO or vehicle (− and open bars). Representative agarose gels showing PCR analysis of PPARγ, UCP1, and β-actin mRNA content on day 1, 4, and 8 after the beginning of the treatment are shown in A and B (top panels). The abundance of PPARγ and UCP1 mRNA was normalized to arbitrary units by assigning the value of 1 to the PPARγ and UCP1 levels on day 8 of GSNO treatment (bottom; $n=4$; $^*P<0.01$ versus mRNA levels on day 1; $^{**}P<0.001$ versus untreated cells). C (top panel) shows a representative agarose gel showing the PCR analysis of PPARγ and β-actin mRNA content in brown fat cells proliferating in culture (day 4) untreated or treated with 300 μM GSNO for 2 h. Bottom; Densitometric analysis of the abundance of PPARγ mRNA normalized to arbitrary units by assigning the value of 1 to the PPARγ mRNA levels with GSNO. $^*P<0.001$ versus untreated cells.

levels by means of densitometry (Figure 2B). Chronic treatment with 300 μM GSNO stimulated PPARγ expression (Figure 2A), and brought forward the appearance of UCP1 (Figure 2B). More interestingly, PPARγ expression in undifferentiated proliferating cells (day 4) was markedly increased after only 2 h of treatment with 300 μM GSNO (Figure 2C). Similar results were obtained also with SNAP. On the other hand, acute GSNO treatment was not able to induce UCP1 expression either in undifferentiated or differentiated cells (no signals in undifferentiated cells also after 2 h of treatment with 300 μM GSNO; 1.06 ± 0.11 versus 1.0 ± 0.12 arbitrary units, obtained by densitometric analysis by assigning the value of 1.0 to the UCP1 mRNA levels without GSNO in differentiated cells). Taken together, these results indicate that, under appropriate experimental conditions, NO is capable of inhibiting cell proliferation and modulating cell differentiation through PPARγ induction.

Effects of 8-bromo-cyclic GMP on cell proliferation and differentiation

In many cell types, the effects of NO on proliferation and/or differentiation are known to take place through the activation of soluble guanylyl cyclase and subsequently increased cyclic GMP generation (Ignarro, 1992). In order to investigate the role of this cyclic nucleotide in the control of proliferation and differentiation by NO in brown adipocytes, we investigated the effects of the membrane-permeable cyclic GMP analogue, 8-bromo-cyclic GMP, as well as those of the selective inhibitor of NO-sensitive guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). The results are shown in Figure 3. Chronic treatment with different concentrations (100–1000 μM) of 8-bromo-cyclic GMP did not affect cell number. Figure 3A shows the lack of effect on cell proliferation of 8-bromo-cyclic GMP, (300 μM), a concentration known to be able to reproduce the effect of NO donors on proliferation in other cell types (see Sciorati *et al*., 1997). Figure 3A also shows that the inhibitory effect of GSNO on cell proliferation was not modified by ODQ (1 μM). These findings suggest that the NO-induced arrest of brown fat cell proliferation is a cyclic GMP-independent process.

Similarly, chronic treatment with different concentrations (100–1000 μM) of 8-bromo-cyclic GMP alone did not increase either UCP1 or PPARγ expression (Figure 3B reports the results obtained with 300 μM 8-bromo-cyclic GMP), suggesting that also the cell differentiation programme could be a cyclic GMP-independent process. However, chronic treatment with 1 μM ODQ greatly reduced the increase in UCP1 and PPARγ gene expression observed after treatment with NO donors (Figure 3B). This suggests that the differentiation programme is a cyclic GMP-dependent process in brown fat cells that can be triggered by NO only after the cell proliferation has been arrested by NO itself. Overall, these results indicate that NO can influence brown fat cell proliferation and differentiation by means of complex signalling pathways: thus inhibition of cell proliferation occurs *via* one or more cyclic GMP-independent mechanism(s), whereas a cyclic GMP-dependent step can influence differentiation.

Discussion

In the present work, we have used precursor brown fat cells in culture to study the effect of NO on rat brown fat growth. When the proliferating immature cells were treated chronically from day 1 to day 8, NO donors, such as SNAP and GSNO markedly inhibited their proliferation. Furthermore, chronic treatment with L-NAME, the selective inhibitor of NOS, stimulated cell proliferation. Exogenously added 8-bromo-cyclic GMP did not have the same effect as NO on brown fat cell proliferation. This suggests that the messenger acts through cyclic GMP-independent mechanism(s). Our results also suggest that chronic treatment of cultured brown adipocytes with NO donors weakly inhibits the mitochondrial conversion of MTT (which is usually employed as a cell proliferation assay), even though this treatment markedly slows down cell proliferation. This may indicate that mitochondriogenesis has proceeded and that the single cells are markedly differentiated. This was further confirmed by the stimulatory effect of NO on the expression of genes that are involved in differentiation of brown adipocytes, such as PPARγ and UCP1.

The growth arrest is a necessary step for brown fat cell differentiation. Indeed, in actively proliferating cells, such as after chronic treatment with L-NAME, mitochondriogenesis is blunted. Similarly, the chronic administration of different concentrations of 8-bromo-cyclic GMP, which does not inhibit cell proliferation, did not affect cell differentiation as measured by PPARγ and UCP1 gene expression. On the other hand, the administration of NO, which is able *per se* to inhibit proliferation, in the presence of ODQ, a selective inhibitor of NO-stimulated guanylyl cyclase, led to a considerable decrease in the induction of PPARγ and UCP1 gene expression. Thus, only the combination of cyclic GMP-dependent and -independent signals seemed to influence the differentiation programme. Taken together, these data are consistent with at least two hypotheses that are not necessarily mutually exclusive: (i) the cyclic GMP-independent, NO-induced arrest of proliferation is absolutely necessary for NO to enhance the differentiation process; (ii) cyclic GMP-independent events occur during differentiation concomitantly with the NO

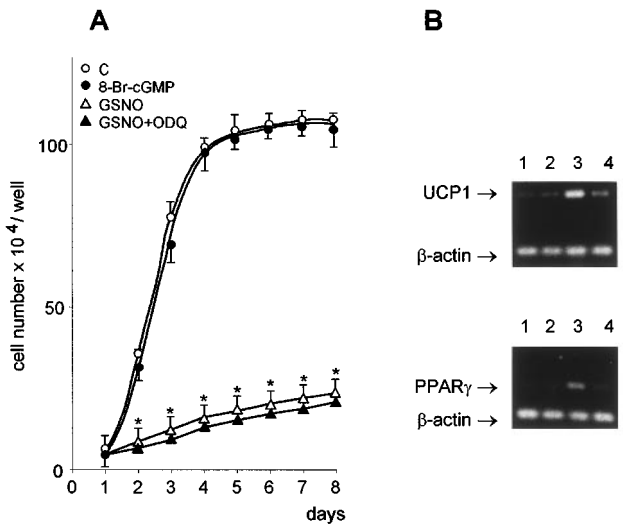

Figure 3 Effects of 8-bromo-cyclic GMP and ODQ on cell number and PPARγ and UCP1 mRNA levels in cultured brown fat cells. (A) The cells were daily treated with vehicle (C, controls) or with 300 μM 8-bromo-cyclic GMP, 300 μM GSNO or 300 μM GSNO plus 1 μM ODQ and their number was determined. ODQ did not have any effect *per se*. (B) Representative agarose gel showing PCR analysis for UCP1, PPARγ, and β-actin content in daily treated cells proliferating in culture on day 4 after 2 h treatment with 300 μM 8-bromo-cyclic GMP (lane 2) or GSNO (lane 3), or 300 μM GSNO plus 1 μM ODQ (lane 4). Lane 1, cells treated with vehicle.

mediated induction of PPARγ and UCP1 expression *via* cyclic GMP-dependent pathway(s). Future studies are needed to clarify this issue.

NO has been reported to affect cell proliferation in different settings. It inhibits tumour cell growth and is cytostatic or cytocidal for certain microorganisms (Nathan, 1992). NO has been shown to inhibit lymphocyte proliferation (Albina & Henry, 1991). NO also affects cellular progression through the cell cycle of different cell types (Takagy *et al*., 1994; Sciorati *et al*., 1997). In addition, Peunova & Enikolopov (1995) showed that the induction of NOS enzyme by nerve growth factor is a necessary event to arrest the growth and to allow the neuronal differentiation of PC12 cells. Unlike in PC12 cells, in which the effect of NO on differentiation seems to be secondary to the arrest of proliferation, our results clearly indicate that NO directly and distinctly affects both the proliferation and differentiation of brown fat cells. In this respect, Hikiji *et al*. (1997) have recently reported a direct action of NO on osteoblastic differentiation.

The stimulatory effect of NO on UCP1 and PPARγ gene expression, that is described here for the first time, is relevant for different reasons. Indeed, UCP1 has been shown to be a very late phenotypic differentiation marker in brown fat cells (Shima *et al*., 1994), and our study accordingly found that only chronic (and not acute) exposure to NO donors increased UCP1 expression, thus indicating that the NO effect may be secondary to brown fat cell differentiation. Similarly, it was very exciting to discover that not only the prolonged, but also the acute (2 h) exposure of proliferating brown fat cells to NO donors increased the expression of the very early PPARγ adipocyte differentiation gene, a member of the PPAR (peroxisome proliferator activated receptor) subfamily of nuclear hormone receptors (Tontonoz *et al*., 1994a; Chwala *et al*., 1994). This receptor is induced at a very early stage of adipose cell differentiation, and is present in preadipocytes at higher levels than in other fibroblastic cells (Yeh & McKnight, 1995). PPARγ appears to function both as a direct regulator of many fat-specific genes, and as a 'master' gene triggering the entire programme of adipogenesis (see Spiegelman & Flier, 1996), including UCP1 expression (Sears *et al*., 1996). Future studies will elucidate whether PPARγ and UCP1 (or other members of UCP family, such as UCP2 and UCP3) (Fleury *et al*., 1997; Boss *et al*., 1997) are the only differentiation-linked genes affected by NO in BAT. Of particular interest are previous observations with thiazolidinediones, a new class of insulin-sensitizing drugs now being considered for the therapy of non-insulin dependent diabetes mellitus (Nolan *et al*., 1994), which promote PPARγ expression and activity (Nolan *et al*., 1994; Lehmann *et al*., 1995; Gimble *et al*., 1996), and can induce adipogenesis in cultured fibroblasts (Kleitzien *et al*., 1992; Tontonoz *et al*., 1994b; Formann *et al*., 1995). It will be interesting to find out whether the mechanism of action of these drugs also involves the increased generation of NO.

It is clear from our observations and from those reported by other investigators that NO can play multiple physiologic and pathophysiologic roles affecting brown fat cell proliferation and differentiation. NO affects brown fat physiology at different levels. Thus NO may mediate increased blood flow to BAT following noradrenergic stimulation (Nagashima *et al*., 1993; Uchida *et al*., 1994). Our work shows that NO also affects the proliferation and differentiation of brown adipocytes themselves. It remains to be established whether therapeutic modulation of NO production and/or NO effects *in vivo* might affect thermogenesis and, thus, energy expenditure in pathological conditions.

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