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Nitric oxide stimulates PC12 cell proliferation via cGMP and inhibits at higher concentrations mainly via energy depletion

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Abstract

We investigated the mechanisms by which nitric oxide (NO) from an NO donor (DETA/NO) regulates proliferation of pheochromocytoma PC12 cells. The NO donor stimulated proliferation at low concentrations, but reversibly and completely inhibited proliferation at higher concentrations. The stimulation (but not the inhibition) of proliferation was apparently due to NO stimulation of soluble guanylate cyclase to produce cGMP, as it was prevented by a specific cyclase inhibitor (ODQ), and replicated by a cell-permeable form of cGMP. The NO-induced cytostasis was not reversed by inhibitors of MEK kinase or poly(ADP-ribose)polymerase, or by treatments that bypass inhibition of ribonucleotide reductase or ornithine decarboxylase. Cytostatic concentrations of DETA/NO strongly inhibited respiration of PC12 cells, and specific respiratory inhibitors (rotenone, myxothiazol, or azide) caused complete cytostasis. Uridine and pyruvate reversed the cytostasis induced by the specific respiratory inhibitors, but not that induced by DETA/NO. However, the combination of uridine, pyruvate, and *N*-acetyl-cysteine did reverse DETA/NO-induced cytostasis. DETA/NO strongly and progressively inhibited glycolysis measured by glucose consumption, lactate production, and ATP level, and a specific glycolytic inhibitor (5 mM 2-deoxy-D-glucose) caused complete cytostasis. Our results indicate that NO at low concentrations increases cell proliferation via cGMP, while high concentrations of NO block proliferation via inhibition of both glycolysis and respiration, causing energy depletion.

Keywords: PC12; cGMP; Nitrogen monoxide; Mitochondria; Cytostasis; Glycolysis

Nitric oxide (NO) has been reported to inhibit cell proliferation in some cell types and conditions, but stimulate cell proliferation in others. The relative dependence of stimulation and inhibition on NO concentration and conditions remains unclear, as do the mechanisms involved. The multiple effects of NO on cell proliferation are important for a number of reasons, including: (i) NO appears to be a physiological regulator of proliferation, particularly in the CNS [1,2] and vasculature [3,4], (ii) NO-induced cytostasis of developing neurons may be required for their differentiation [5], (iii) NO from activated macrophages limits the proliferation of pathogens and tumour cells, but NO may also promote the growth of some tumours [6], and (iv) NO donor drugs are used in a wide range of medical conditions, and NO synthase gene therapies are being introduced [7]. Thus, it is important to understand how NO controls proliferation.

NO is thought to be a major regulator of the proliferation and differentiation of developing neurons in the brain, via inhibition of proliferation [1,2,8,9]. The mechanisms responsible for NO-induced suppression of proliferation of neuronal precursor have not been characterised. However, research on PC12 cells (used as a model of neurons or neuronal precursors) showed that endogenous or exogenous NO blocked proliferation, and this block on proliferation was required for subsequent differentiation [5,10]. Whether NO could stimulate proliferation of PC12 or neuronal precursors was not reported.

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In culture it was shown some time ago that NO from NO donors or from NO-producing macrophages could inhibit the proliferation of tumour cells or tumour-derived cell lines [11]. Indeed, this was one of the lines of research that lead to the discovery of the biological role of NO in the body, and also lead to the idea that activated macrophages in tumours suppressed tumour cell growth via NO [12,13]. NO and macrophage-induced cytostasis and cytotoxicity of tumour cells was initially attributed to inhibition of mitochondrial respiration due to irreversible inhibition of mitochondrial aconitase, complex I, and complexes II/III [12]. Later it was found that NO could also reversibly inhibit complex IV (cytochrome *c* oxidase) [14,15], and that activated macrophages could immediately inhibit the respiration of co-incubated tumour cells via NO inhibition of complex IV [16]. But whether NO inhibition of respiration could account for NO-induced cytostasis has not been directly tested.

Subsequently NO- and macrophage-induced cytostasis was attributed to inhibition of ribonucleotide reductase [17,18], an enzyme required for deoxynucleotide synthesis. Thus, exogenously added deoxynucleosides could partially overcome this cytostasis [17,18]. However, although the level of cytostasis correlated with the level of reductase inhibition for the first few hours after exposure to NO, it did not do so after longer exposure to NO or in cells that were also thiol depleted, indicating that other mechanisms are involved in these conditions [18,19]. NO-induced cytostasis in human breast cancer cells was associated with the down-regulation of cyclin D1 and hypophosphorylation of the retinoblastoma protein [20], but this tells us little about the upstream mechanisms. S-nitrosation of ornithine decarboxylase has been implicated in cytostasis of cell lines by the finding that exogenous spermine and putrescine (products of the ornithine decarboxylase pathway) can reverse the NO-induced cytostasis [21]. S-nitrosation and consequent inhibition of caspases has been suggested to mediate NO-induced inhibition of T-cell proliferation [22].

We initially set out to test whether NO-induced cytostasis was mediated by NO inhibition of mitochondrial respiration or other mechanisms. However, we found that the effects of NO on PC12 cell proliferation were biphasic: stimulation at low levels and inhibition at higher levels, with an overlap in the dose response of the two effects. We then found that the stimulation was mediated by cGMP, while the inhibition was mediated by energy depletion resulting from blockage of both respiration and glycolysis.

Materials and methods

Materials

Diethylenetriamine-nitric oxide adduct (DETA/NO) was from RBI, Dulbecco's modified Eagle's medium (DMEM) was from Gibco-BRL, 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ) was from RBI, and [3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2*H*)-isoquinolinone] (DPQ) and *n*-acetylcysteine (NAC) were from Sigma.

Cell cultures

Rat pheochromacytoma cell line (PC12) purchased from American Type Culture Collection (ATCC) was cultured on collagen-coated 24-well plates (density 1.0×10^4 cells/cm^2) in DMEM supplemented with heat-inactivated horse serum (10% v/v) and foetal bovine serum (5% v/v). All cell cultures contained 100 U/ml penicillin, 100 mg/ml streptomycin and were maintained at 37 °C in 5% CO_2 atmosphere.

PC12 cells were exposed to 25–250 μM DETA/NO for 24 or 48 h in the presence or absence of 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ, 10 μM), an inhibitor of soluble guanylate cyclase. In separate experiments, cells were also exposed to a range of other agents for 24 or 48 h as indicated in figure legends.

The murine monocyte-derived macrophage cell line (J774) was from the laboratory of Salvador Moncada (UCL, London). Inducible NO synthase (iNOS) was activated in the macrophages (approximately 2×10^5 cells/ml) by adding *Salmonella typhimurium* lipopolysaccharide (LPS, 10 μg/ml) and interferon-γ (IFN-γ, mouse recombinant, 50 U/ml), and incubating the cells at 37 °C for 18 h in stirrer culture with arginase (0.2 U/ml, present to limit NO-induced death of macrophages). For the separated co-culture system, activated macrophages after 18 h were spun down, resuspended, plated (density 4.0×10^4/cm^2) on inserts with 0.4-μm pore size (Falcon), and placed above the underlying PC12 cells (density 1.0×10^4 cells/cm^2) sharing the same culture medium in the presence or absence of aminoguanidine (400 μM).

Measurements of cell proliferation

Cell proliferation was assessed by counting the number of the cells per field (four fields per well; 3 wells per treatment) in every experiment just before the treatment and compared with the number of the cells after 24 or 48 h of exposure to DETA/NO or myxothiazol. The initial number of cells per field at time 0 was 42 ± 11. Cell viability was estimated by trypan blue exclusion (0.4%, light microscope) or propidium iodide exclusion (necrosis, fluorescent microscope) or chromatin condensation visualised with Hoechst 33342 (fluorescent microscope).

Measurement of NO generation and oxygen consumption

Measurement of NO levels released by NO donor (DETA/NO) and oxygen consumption in the presence of PC12 cells was performed using a Clark-type NO electrode (World Precision Instruments) inserted through the top of an oxygen electrode chamber (Rank Brothers, Bottisham), permitting simultaneous measurement of NO and oxygen levels. Experiments were performed in 1 ml of Krebs–Hepes buffer consisting of: 1.5 mM $CaCl_2$, 5.6 mM glucose, 10 mM Hepes, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4$, and 118 mM NaCl, pH 7.4, at 37 °C, in the presence or absence

of PC12 cells (approximately 1.5×10^6 cells), stirred continuously at a constant speed. The rate of oxygen consumption by PC12 cell was calculated before and after the addition of DETA/NO (range between 50 and 200 μM) acutely or after 24 h of incubation, and expressed in nmol O/min/number of cells. The NO electrode was calibrated by adding aliquots of NO-saturated water assumed to contain 2 mM NO.

Measurement of glucose, lactate, and ATP

Glucose levels in the medium were determined using a Glucose kit (Sigma Diagnostic) containing glucose oxidase, peroxidase, and *o*-dianisidine, by the method of Raabo and Terkildsen [23]. Aliquots of deproteinised culture medium were also assayed for lactate using a L-lactic acid kit (Boehringer–Mannheim), monitoring the oxidation of L-lactic acid to pyruvate by NAD in the presence of L-lactate dehydrogenase. Cellular ATP was determined luminometrically (Jade luminometer, Labtech International) using an ATP Bioluminescence Assay Kit (Boehringer–Mannheim) based on the light-emitting luciferase-catalysed oxidation of luciferin.

Statistics

All experiments were performed on at least three separate cell cultures on different occasions, and on each occasion each treatment/experiment was performed at least three times. Statistical differences were tested using ANOVA.

Results

Nitric oxide stimulates cell proliferation at low concentrations via a cGMP-dependent mechanism and inhibits proliferation at high concentrations

To test how NO affects cell proliferation, PC12 cells were exposed to the NO donor (DETA/NO) at a range of concentrations for 24 or 48 h. Exposure of cells to DETA/NO for 24 h at low concentrations (25–50 μM) significantly stimulated proliferation (Fig. 1A). These low concentrations of DETA/NO were found to produce between 20 and 100 nM of NO as measured by the NO electrode (data not shown). Higher concentrations of DETA/NO (150–250 μM) produced between 200 and 600 nM NO and inhibited proliferation (Fig. 1A). Similar results were obtained after 48 h of incubation (Fig. 1B). Twenty-four or 48 h of exposure to 200 μM DETA/NO induced complete cytostasis, without causing any detectable necrosis (as indicated by trypan blue staining of cells or propidium iodide staining of nuclei) or apoptosis (as indicated by condensation or fragmentation of Hoescht 33342-stained chromatin). The inhibitory effect of NO on proliferation was reversible: incubation of PC12 cells with 200 μM DETA/NO caused complete cytostasis, but after replacement of the DETA/NO-containing media with the same culture medium lacking DETA/NO, the rate of proliferation returned to 55.1 ± 9.0% of the control rate of proliferation after 48 h.

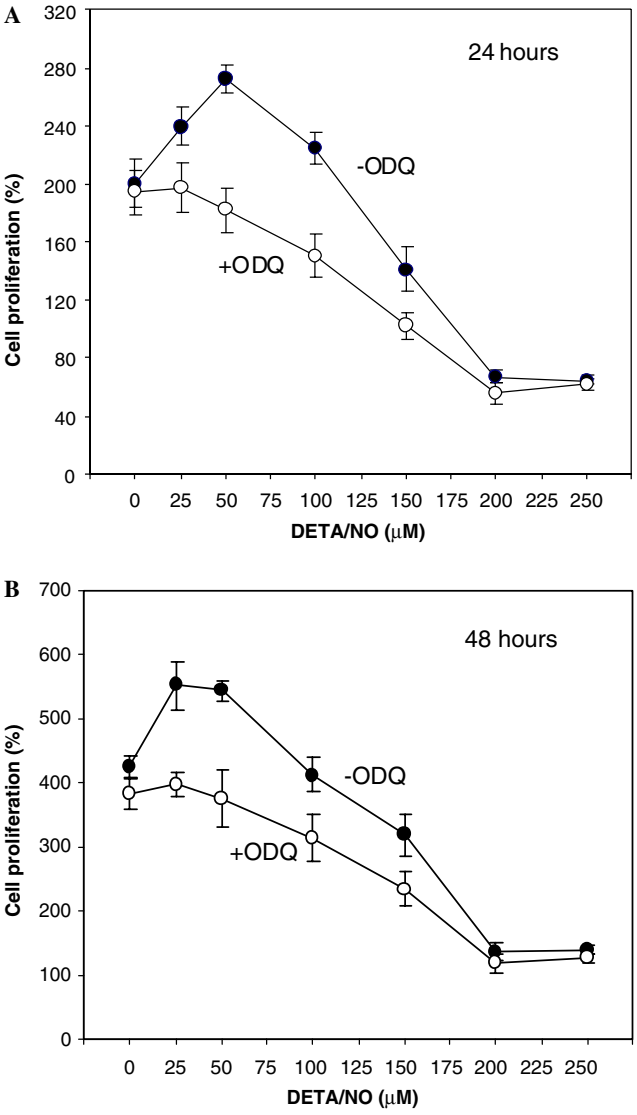

Fig. 1. The effect of NO donor (DETA/NO) on the proliferation of PC12 cells after (A) 24 h and (B) 48 h of incubation, in the presence or absence of ODQ (10 μM), a selective inhibitor of soluble guanylate cyclase. The number of cells was counted and expressed as the percentage of the cells present at time zero (see Materials and methods) and values represent means ± SD (bars) of the means in at least three independent experiments.

To test whether DETA/NO acts on proliferation via NO stimulation of soluble guanylate cyclase and increased level of cGMP, we used ODQ, a selective inhibitor of soluble guanylate cyclase. Ten micromolar ODQ was added to the cells before the DETA/NO, and was found to completely prevent the DETA/NO-induced stimulation of proliferation, without having any effect on the DETA/NO-induced inhibition of proliferation (Figs. 1A and B). Thus, it would appear that NO stimulates proliferation via soluble guanylate cyclase, but the inhibition of proliferation is not mediated by soluble guanylate cyclase. To test whether cGMP could directly stimulate proliferation, we added a cell-

permeable form of cGMP (dibutryl-cGMP) to the cells. Dibutryl-cGMP (0.5 mM) stimulated the proliferation rate of PC12 cells by 37.3 ± 11.4% (of control untreated cells) after 24 h of exposure. Thus, a rise in cGMP is necessary and sufficient to stimulate proliferation.

NO-induced cytostasis is not mediated by ribonucleotide reductase, ornithine decarboxylase, p42/44 MAP kinase or PARP

NO-induced cytostasis has been suggested to be mediated by inhibition of ribonucleotide reductase. Cytostasis due to inhibition of ribonucleotide reductase (a rate-limiting enzyme in deoxyribonucleotide synthesis) is reversible by addition of deoxyribonucleosides [17–19]. However, we found that cytostasis of PC12 cells induced by 200 μM DETA/NO was unaffected by exogenous deoxyribonucleosides (Fig. 2), indicating that inhibition of ribonucleotide reductase was not responsible for the cytostasis.

S-nitrosation of ornithine decarboxylase has been implicated in cytostasis of Caco-2 tumour cell lines and vascular smooth muscle cells by the finding that exogenous spermine and putrescine (products of the ornithine decarboxylase pathway) can reverse the NO-induced cytostasis [21,24]. However, we found only a small reversal of cytostasis by spermine, but no reversal by putrescine (Fig. 2).

In vascular smooth muscle cells, NO-induced cytostasis may be partially prevented by the p42/44 MAP kinase pathway inhibitor PD 098059, apparently by preventing NO-induced expression of p21(WAF1), the cyclin-dependent kinase inhibitor [25,26]. However, using the same inhibitor at similar or higher concentration in PC12 cells we found no effect on NO-induced cytostasis (cell numbers 24 h after treatment: 100 ± 5% in control and 38 ± 4% with 200 μM DETA/NO in absence of PD 098059; and in the presence of 50 μM PD 098059 85 ± 3% in control and 38 ± 3% with DETA/NO; means ± SD of means of three separate experiments).

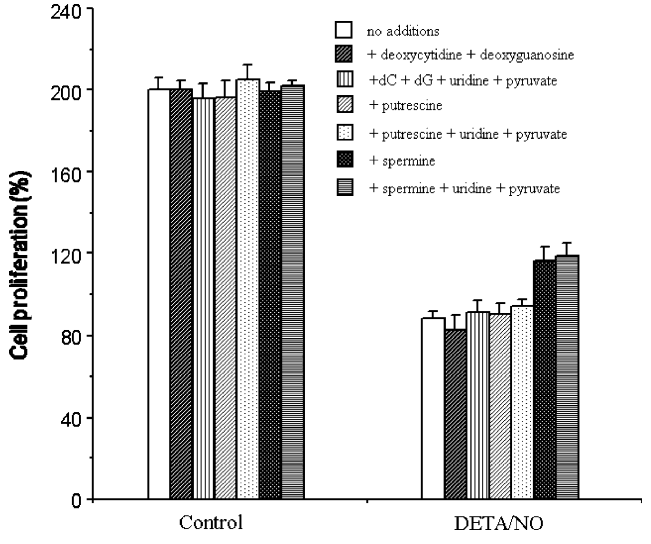

Fig. 2. The presence of various deoxyribonucleosides or polyamines (alone or in combination) did not prevented DETA/NO (200 μM)-induced inhibition of PC12 cells proliferation (24 h incubation). Concentrations used were: 100 μM deoxycytidine, 100 μM deoxyguanosine, 50 μg/ml uridine, 100 μg/ml pyruvate, 100 μM putrescine, 300 μM spermine, and 300 μM spermidine. The data present means ± SD of the means (bars) of three independent experiments.

NO can activate poly(ADP-ribose)polymerase (PARP), and this activation can mediate NO-induced cell death in some cells [27] and has been suggested to mediate NO-induced cytostasis of hepatocytes [28]. However, we found that a PARP inhibitor (DPQ) had no effect on NO-induced cytostasis of PC12 cells (cell numbers 24 h after treatment: 100 ± 17% in control and 43 ± 7% with 200 μM DETA/NO in absence of DPQ; and in the presence of 10 μM DPQ 99 ± 12% in control and 39 ± 4% with DETA/NO; means ± SD of means of three separate experiments), suggesting that PARP has no role in the NO-induced cytostasis of these cells.

NO inhibits cellular respiration and respiratory inhibitors cause cytostasis

NO has been suggested to induce cytostasis via inhibition of mitochondrial respiration. We therefore determined the extent to which DETA/NO, at the concentrations that inhibit PC12 cell proliferation (150–250 μM) also inhibited cellular respiration. We measured the oxygen consumption of PC12 cells after acute or prolonged exposure (24 h) to DETA/NO at these concentrations. Addition of 200 μM DETA/NO caused a rapid and potent inhibition of PC12 cells respiration rate (5.2 ± 1.1 nmol O/min/10^6 cells before adding DETA/NO; 1.1 ± 0.5 nmol O/min/10^6 cells 10 min after adding DETA/NO), and this inhibition was completely reversed by the addition of an NO scavenger oxyhaemoglobin (rate increased to 5.0 ± 0.8 nmol O/min/10^6 cells). After 24 h incubation of PC12 cells with DETA/NO (200 μM) irreversible inhibition of respiration was observed: the rate of oxygen consumption before and after addition of oxyhaemoglobin was almost the same, 1.8 ± 0.4 and 2.0 ± 0.3 nmol O/min/10^6 cells, respectively.

Since NO inhibited respiration, we wanted to test whether specific respiratory inhibitors would cause cytostasis. Myxothiazol is a specific inhibitor of complex III of the mitochondrial respiratory chain, and we verified using the oxygen electrode that 1 μM myxothiazol caused complete inhibition of PC12 cell respiration (data not shown). Complete inhibition of cell proliferation (without causing any cell death) was observed after exposure of PC12 cells (Fig. 3) to 0.25–2 μM myxothiazol (PC12 cell numbers per field: 58 ± 6 at zero time, 140 ± 9 at 24 h in absence of myxothiazol, 94 ± 9 with 0.25 μM myxothiazol, 60 ± 6 with 0.5 μM myxothiazol, 58 ± 6 with 1.0 μM myxothiazol, 60 ± 7 with 2.0 μM myxothiazol; means ± SD of means of three separate experiments). One micromolar rotenone (a specific inhibitor of mitochondrial complex I) and 1–2 mM azide (an inhibitor of mitochondrial respiratory complex IV) also caused complete cytostasis without significant cell death 24 h after addition (PC12 cell

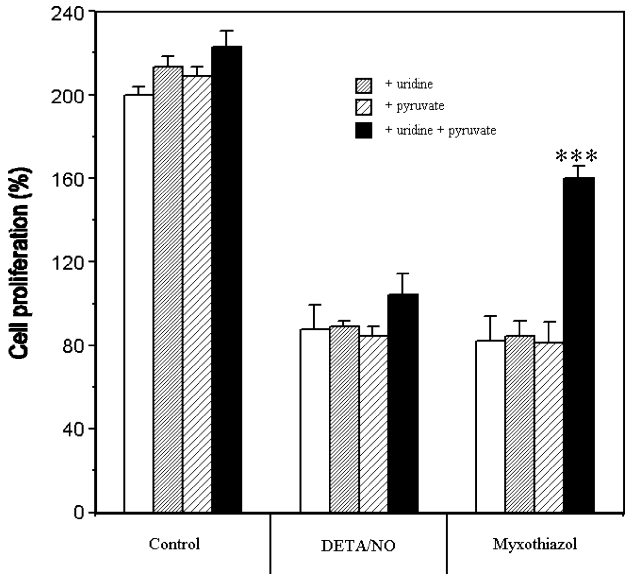

Fig. 3. The inhibition of PC12 cell proliferation induced by myxothiazol (2 μM), but not DETA/NO (200 μM), was prevented by uridine (50 μg/ml) and pyruvate (100 μg/ml) (24 h incubation). The data present means ± SD of the means of three independent experiments. Proliferation rate in the presence of myxothiazol + uridine + pyruvate was significantly different (***$p < 0.001$) from myxothiazol only.

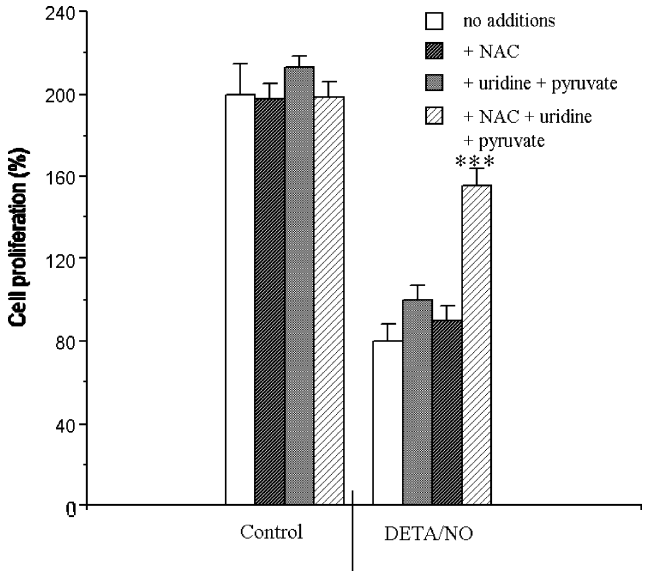

Fig. 4. The effect of DETA/NO (200 μM) on the proliferation of PC12 cells (24 h incubation) in the absence and presence of NAC (1 mM) or uridine (50 μg/ml) plus pyruvate (100 μg/ml), or NAC together with uridine and pyruvate. Values represent means ± SD of the means of at least three independent experiments. Proliferation rate in the presence of DETA/NO + NAC + uridine + pyruvate was significantly different (***$p < 0.001$) from DETA/NO only.

numbers 24 h after treatment: 100 ± 2% with no additions, 42 ± 7% with 1 μM rotenone; 40 ± 4% with 1 mM azide, 38 ± 4% with 2 mM azide). Since specific respiratory chain inhibitors blocked proliferation and the NO donor inhibited cellular respiration over the same range of concentrations that blocked proliferation, then the NO donor must block proliferation via respiratory inhibition, unless NO does something else to prevent this linkage.

Pyruvate plus uridine has previously been shown to prevent a block on cell proliferation induced by eliminating mitochondrial DNA from cells [29]. We found that exogenous pyruvate and uridine could partly reverse the cytostasis induced by the specific respiratory inhibitor myxothiazol, but pyruvate and uridine were unable or poorly able to reverse the cytostasis induced by NO (Fig. 3). Pyruvate and uridine also partly prevented the cytostasis induced by specific respiratory inhibitors rotenone and azide (PC12 cell numbers 24 h after treatment: 100 ± 2% with no additions, 42 ± 7% with 1 μM rotenone; 40 ± 4% with 1 mM azide, 38 ± 4% with 2 mM azide in the absence of uridine and pyruvate; and in the presence of 50 μg/ml uridine and 100 μg/ml pyruvate: 104 ± 2% with no further additions, 62 ± 5% with 1 μM rotenone; 49 ± 5% with 1 mM azide, 39 ± 5% with 2 mM azide). Thus, NO must be doing something in addition to respiratory inhibition to induce cytostasis.

We tested various combinations of exogenous substrates capable of reversing cytostasis induced by particular mechanisms to test whether a combination of mechanisms was involved in NO-induced cytostasis. Uridine and pyruvate plus spermine or putrescine or deoxyribonucleotides (Fig. 2), or uridine and pyruvate plus ODQ (data not shown) were not synergistic in relieving cytostasis. However, we found that uridine and pyruvate plus *N*-acetyl-cysteine (NAC) was capable of relieving NO-induced cytostasis (Fig. 4), even though pyruvate plus uridine or NAC alone had no effect on cytostasis. NAC at the concentrations used had no significant affect on NO levels generated from DETA/NO measured by NO electrode (data not shown). NAC protects cellular glutathione levels, and thus potentially prevents NO-induced inhibition of glycolysis [30]. So we investigated whether NO-induced cytostasis might be related to inhibition of glycolysis, as previously suggested [19].

NO inhibits glycolysis; inhibition of glycolysis causes cytostasis

To determine whether a cytostatic level of DETA/NO inhibits glycolysis we measured glucose and lactate levels in the culture of PC12 cells exposed to 200 μM DETA/NO for 24 and 48 h. DETA/NO caused a strong inhibition of both glucose consumption (Fig. 5A) and lactate production (Fig. 5B). However, NO inhibition of glycolysis is complicated (a) by the probability that NO inhibition of respiration will stimulate glycolysis, and (b) by fact that inhibition of proliferation will decrease the number of cells and thus the overall glycolytic rate. We therefore compared the effect of NO on glycolysis to the effect of the specific respiratory inhibitor myxothiazol, which like DETA/NO inhibits respiration and causes complete cytostasis. If DETA/NO affects the glycolytic rate solely by inhibiting respiration then it should have the same effect as myxothiazol. However, DETA/NO caused a substantially greater inhibition of

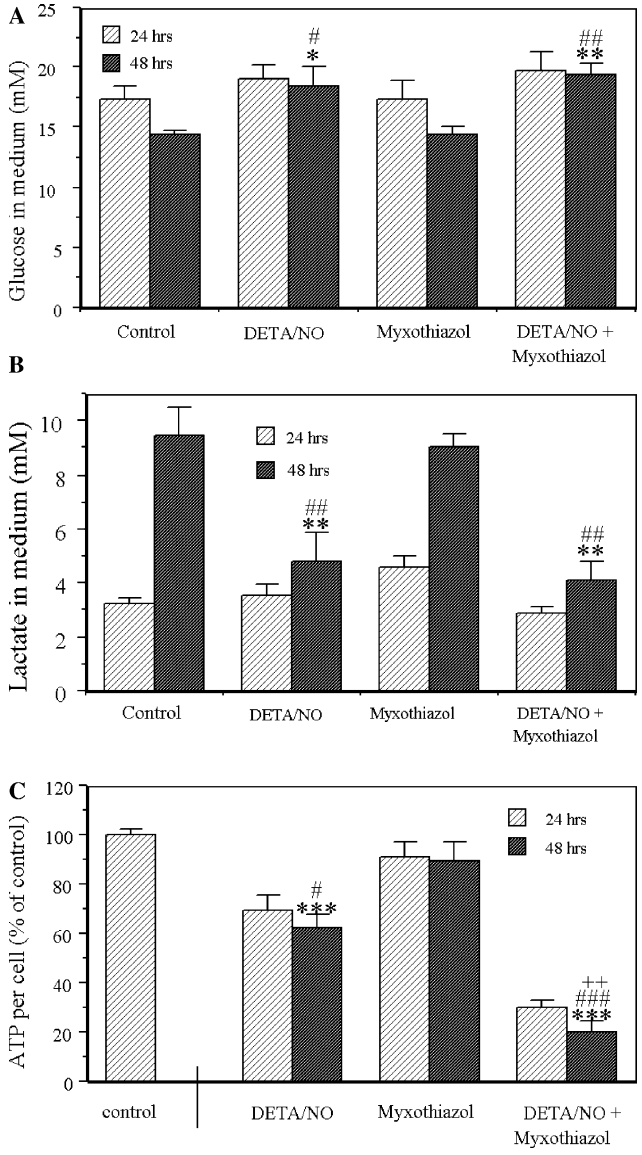

Fig. 5. The effect of DETA/NO (200 μM) and myxothiazol (2 μM) or DETA/NO plus myxothiazol together on glucose (A) and lactate (B) levels in the medium, and ATP (C) levels in the PC12 cells after 24 or 48 h incubation. The level of glucose in the medium before additions was 23.0 ± 0.1 mM. The level of lactate in the medium was 1.4 ± 0.1 mM before additions. The ATP data are expressed as % of control (100% 1.6 ± 0.2 nmol of ATP/10^6 cells). Values represent means ± SD (bars) of the means of at least three independent experiments. Significant differences are indicated for the 48 h data: ∗ from control, # from myxothiazol treated, and + from DETA/NO treated; where $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

glucose consumption and lactate production than myxothiazol, the inhibition being more marked in the second 24 h of exposure to DETA/NO (Figs. 5A and B). When myxothiazol was added together with DETA/NO, there was a large inhibition of lactate production and glucose consumption (particularly in the second 24 h) relative to myxothiazol alone, but little effect compared to DETA/NO alone (Figs. 5A and B). Thus, DETA/NO strongly and progressively inhibited glycolysis independent of its inhibition of respiration or proliferation.

If NO inhibits glycolysis and/or respiration it should decrease the cellular ATP level. Fig. 5C shows that 200 μM DETA/NO decreased the cellular ATP level to a substantially greater degree than the specific respiratory inhibitor myxothiazol, and when myxothiazol and DETA/NO were given together they caused a further progressive fall in ATP levels. This is consistent with NO causing a progressive inhibition of glycolysis resulting in ATP depletion, which might then be responsible for cytostasis. Myxothiazol added together with DETA/NO depleted ATP more with DETA/NO alone, but this may be simply because myxothiazol completely inhibited respiration while DETA/NO inhibition of respiration was partial (about 80%, see above).

To test whether inhibition of glycolysis alone could cause cytostasis we added an inhibitor of glycolysis, 5 mM deoxy-D-glucose, and found that this caused a very strong inhibition of PC12 proliferation. Mean cell numbers per field (±SEM of three independent experiments) at 0, 24, 48, and 72 h were 10.2 (±0.3), 24.5 (±1.5), 77.3 (±15.2), and 206.3 (±45.3), and in the presence of 5 mM deoxyglucose were 10.2 (±0.3), 12.1 (±0.27), 16.2 (±3.7), and 19.4 (±9.8). The deoxyglucose also strongly inhibited lactate production (data not shown) and decreased ATP levels to a similar extent as that caused by 200 μM DETA/NO (ATP per cell: 100 ± 3% without deoxyglucose; 61 ± 2% 24 h after adding 5 mM deoxyglucose; 54 ± 3% 48 h after adding 5 mM deoxyglucose). Depleting the medium of glucose also caused strong, progressive inhibition of proliferation that was reversed by adding 20 mM glucose to the depleted medium (data not shown). Thus, inhibition of glycolysis is sufficient to inhibit proliferation.

Activated macrophages induce cytostasis of PC12 cells via iNOS

Although we have shown that a NO donor (DETA/NO) can produce sufficient NO to induce cytostasis of PC12 cells, it is unclear whether sufficient NO can be produced physiologically or pathophysiologically from NO synthases to induce cytostasis. To examine this we co-cultured PC12 cells with a macrophage cell line (J774), inflammatory activated with LPS and interferon-γ to express iNOS, but separated from the PC12 cells by an insert permeable to small molecules (see Materials and methods). The proliferation of the PC12 cells co-cultured with activated macrophages was strongly inhibited, and this inhibition was prevented by the addition of aminoguanidine, an inhibitor of iNOS (Fig. 6). This is consistent with activated macrophages producing sufficient NO from iNOS to induce cytostasis of PC12 cells.

Discussion

The NO donor DETA/NONOate had a biphasic effect on proliferation, stimulating at low concentrations (25–50 μM DETA/NO, producing 20–100 nM NO) and inhibiting at high concentrations (150–250 μM, producing 200–600 nM

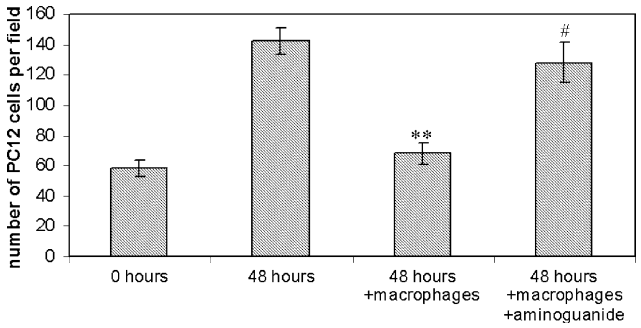

Fig. 6. The effect of LPS/IFN-γ activated macrophages on the proliferation of co-cultured PC12 cells. Activated macrophages co-cultured with PC12 cells (separated by permeable insert, see Materials and methods) caused cytostasis of PC12 cells after 48 h. The inhibition of PC12 cell proliferation by activated macrophages was prevented in the presence of aminoguanidine (400 μM). Numbers of PC12 cells were counted in random fields at 0 and 48 h, cultured either in the absence or presence of activated macrophages. 48 h + macrophages was significantly different ($^{**}p < 0.01$) from 48 h untreated, and 48 h + macrophages + aminoguanidine was significantly different ($^{\#}p < 0.05$) from 48 h + macrophages.

NO). Two hundred micromolar DETA/NONOate caused complete but reversible cytostasis, while higher concentrations (500–1000 μM) caused cell death. The stimulation and inhibition of proliferation were by different (cGMP-dependent and cGMP-independent, respectively) mechanisms, and the dose responses of these different mechanisms to NO level overlapped, so that over a range of concentrations (50–150 μM DETA/NO) NO was both stimulating and inhibiting proliferation (Fig. 1). This overlapping, biphasic response to NO has implications for angiogenesis, neurogenesis, and tumour growth, where there may be tissue gradients of NO.

The stimulation of proliferation by NO is clearly mediated by cGMP in PC12 cells (as previously shown for endothelial cells [4]), as it is completely prevented by ODQ (a specific inhibitor of soluble guanylate cyclase), and is replicated by a cell-permeable form of cGMP.

We investigated several possible causes of NO-induced cytostasis. We found that cytostasis of PC12 cells induced by 200 μM DETA/NONOate was unaffected by exogenous deoxyribonucleosides (Fig. 2), indicating that ribonucleotide reductase was not responsible for the cytostasis. This is consistent with Lepoivre et al. [18] who found that after a brief exposure of tumour cells to NO or macrophages (3–4 h) cytostasis was reversible by deoxyribonucleosides, but after a longer-term exposure to NO (overnight) cytostasis was not reversible by deoxyribonucleosides, indicating that a second mechanism of cytostasis developed over time. Similarly Lemaire et al. [19] found that early cytostasis of K562 tumour cells induced by 3 h exposure to NO donors was reversible by deoxyribonucleosides, but that depleting cellular thiols prior to NO exposure resulted in a cytostasis that was irreversible, and which they ascribed to inhibition of glycolysis. This interpretation may be consistent with our own outlined below.

S-nitrosation of ornithine decarboxylase has been implicated in cytostasis of Caco-2 tumour cell lines and vascular smooth muscle cells by the finding that exogenous spermine and putrescine (products of the ornithine decarboxylase pathway) can reverse the NO-induced cytostasis [21,24]. However, the models of NO-induced cytostasis used in these published studies differ in significant ways from that used by us and other researchers in that low levels of NO donor (1–10 μM SNAP; 3–30 μM DETA/NO) were used for an extended period (8 days), and only a relatively small inhibition of proliferation was observed (around 50%), which was fully reversible by spermine and putrescine. In addition, these authors used a low arginine medium (30–50 μM) that may sensitise the cells to polyamine limitation of proliferation. By contrast we used relatively high levels of NO donor to induce complete cytostasis over 1 or 2 days, and found only a small reversal of cytostasis by spermine, but no reversal by putrescine (Fig. 2). The small reversal of cytostasis by spermine (about 20%) may be consistent with a small contribution of ornithine decarboxylase inhibition to cytostasis, but the lack of effect of putrescine is not consistent. However, it is clear that most of the cytostasis (80–100%) is not reversible by putrescine or spermine, and therefore presumably is not mediated by inhibition of ornithine carboxylase.

In vascular smooth muscle cells, NO-induced cytostasis may be partially prevented by the p42/44 MAP kinase pathway inhibitor PD059098, apparently by preventing NO-induced expression of p21(WAF1), the cyclin-dependent kinase inhibitor [25,26]. However, using the same inhibitor at similar or higher concentration in PC12 cells we found no effect on NO-induced cytostasis. NO can activate PARP, and has been suggested to mediate NO-induced cytostasis of hepatocytes [28]. However, we found that a PARP inhibitor (DPQ) had no effect on NO-induced cytostasis of PC12 cells, suggesting that PARP activation has no role in the NO-induced cytostasis of these cells.

At concentrations that induced cytostasis, NO caused substantial inhibition of cellular respiration in PC12 cells. There was an immediate reversible effect (probably due to inhibition of cytochrome oxidase) and a more slowly developing irreversible effect (probably due to inhibition of complex I) as reported previously [31,32]. A number of specific inhibitors of the respiratory chain (rotenone, myxothiazol, and azide) all caused complete cytostasis without causing significant cell death in PC12 cells. The respiratory-inhibitor-induced cytostasis was partly reversed by exogenous uridine and pyruvate, as has been reported for the cytostasis induced by loss of mitochondrial DNA from cell lines [29]. Uridine is thought to work by bypassing mitochondrial dihydroorotate dehydrogenase, an enzyme of purine synthesis that passes its electrons into the mitochondrial respiratory chain, and is therefore inhibited when the latter is inhibited [33]. The requirement for pyruvate is thought to be linked to oxidising cytoplasmic NADH [29]. It is of some interest (independent of NO) that respiratory inhibition can cause cytostasis, as for example a number of patholo-

gies result in respiratory inhibition, and tumour growth is often limited by hypoxia.

Beuneu et al. [33] have previously suggested that NO might cause cytostasis via NO inhibition of respiration indirectly blocking dihydroorotate dehydrogenase and thus uridine biosynthesis. We found that exogenous pyruvate and uridine could partly reverse the cytostasis induced by specific respiratory inhibitors, but pyruvate and uridine were unable or poorly able to reverse the cytostasis induced by NO. Thus, NO must be doing something in addition to respiratory inhibition to induce cytostasis. Uridine and pyruvate plus spermine, and uridine and pyruvate plus deoxyribonucleotides were not synergistic in relieving cytostasis. However, we found that uridine and pyruvate plus *N*-acetyl-cysteine (NAC) were remarkably synergistic in relieving NO-induced cytostasis (Fig. 4). NAC protects cellular glutathione levels, and thus may prevent NO-induced inhibition of glyceraldehyde-3-phosphate dehydrogenase and glycolysis [19,30,34]. So we investigated whether NO-induced cytostasis might be related to inhibition of glycolysis, as has previously been suggested [19].

DETA/NO did indeed progressively inhibit glycolysis in the cells. As glycolysis and mitochondrial oxidative phosphorylation are the only two means of producing ATP in cells, we would expect inhibition of these processes to decrease cellular ATP levels. Indeed, both myxothiazol and DETA/NO reduced cellular ATP levels, but the reduction caused by DETA/NO was larger than that by myxothiazol, and DETA/NO together with myxothiazol greatly reduced ATP levels compared to myxothiazol alone (Fig. 5). Thus, DETA/NO must be decreasing ATP levels by means in addition to inhibition of respiration: presumably the inhibition of glycolysis. Note that most ATP production in these cells was from glycolysis rather than mitochondria: glycolytic rates were about 52 nmol lactate/min/million cells equivalent to 52 nmol ATP/min/million cells, while the respiration rate was 5.6 nmol O/min/million cells equivalent to about 14 nmol ATP/min/million cells. Thus, about 21% of cellular ATP production was from mitochondria, 79% from glycolysis. This is consistent with the rate of lactate production being roughly double the rate of glucose consumption (Fig. 5), i.e., virtually all the glucose was converted to lactate rather than being oxidised by the mitochondria. That relatively little of the cellular ATP production was derived from mitochondria is also consistent with the finding that the respiratory inhibitor myxothiazol decreased ATP levels relatively little.

Since NO inhibited glycolysis we tested whether specific inhibition of glycolysis by deoxy-D-glucose could cause cytostasis. Five millimolar deoxy-D-glucose caused very strong inhibition of proliferation and lactate production, and decreased ATP levels to a similar extent to that caused by 200 μM DETA/NO. This is strong circumstantial evidence that the NO-induced cytostasis was due to the NO-induced inhibition of glycolysis and the resulting ATP depletion. Glycolysis is required for the proliferation of many cell types [35].

NO inhibition of glycolysis has been attributed to progressive inhibition of glyceraldehyde-3-phosphate dehydrogenase, but this inhibition can be prevented by preventing cellular glutathione with reduced thiols such as *N*-acetyl-cysteine (NAC) [30,34]. If the inhibition of glycolysis by NO is responsible for the cytostasis, then the cytostasis should be reversed by agents that reverse the inhibition of glycolysis. NAC alone was not capable of preventing the NO-induced cytostasis. However, in combination with uridine and pyruvate to overcome the respiratory inhibition-induced cytostasis, NAC was capable of largely reversing the NO-induced cytostasis (Fig. 4). This is consistent with the NO-induced cytostasis being due to both NO-induced inhibition of respiration and glycolysis.

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References

[1] N. Peunova, V. Scheinker, H. Cline, G. Enikolopov, NO is an essential negative regulator of cell proliferation in *Xenopus* brain, J. Neurosci. 21 (2001) 8809–8818.
[2] S.M. Gibbs, Regulation of neuronal proliferation and differentiation by nitric oxide, Mol. Neurobiol. 27 (2003) 107–120.
[3] J.Y. Jeremy, D. Rowe, A.M. Emsley, A.C. Newby, Nitric oxide and the proliferation of vascular smooth muscle cells, Cardiovasc. Res. 43 (1999) 580–594.
[4] A. Parenti, L. Morbidelli, F. Ledda, H.J. Granger, M. Ziche, The bradykinin/B1 receptor promotes angiogenesis by up-regulation of endogenous FGF-2 in endothelium via the nitric oxide synthase pathway, FASEB J. 5 (2001) 1487–1489.
[5] N. Peunova, G. Enikolopov, Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells, Nature 375 (1995) 68–73.
[6] P.K. Lala, C. Chakraborty, Role of nitric oxide in carcinogenesis and tumour progression, Lancet Oncol. 2 (2001) 149–156.
[7] K.G. Raman, R.A. Shapiro, E. Tzeng, M.R. Kibbe, Adenovirus-mediated nitric oxide synthase gene transfer, Methods Mol. Biol. 279 (2004) 225–234.
[8] G. Enikolopov, J. Banerji, B. Kuzin, Nitric oxide and *Drosophila* development, Cell Death Differ. 6 (1999) 956–963.
[9] B. Kuzin, I. Roberts, N. Peunova, G. Enikolopov, Nitric oxide regulates cell proliferation during *Drosophila* development, Cell 87 (1996) 639–649.
[10] W. Poluha, C.M. Schonhoff, K.S. Harrington, M.B. Lachyankar, N.E. Crosbie, D.A. Bulseco, A.H. Ross, A novel, nerve growth factor-activated pathway involving nitric oxide, p53, and p21WAF1 regulates neuronal differentiation of PC12 cells, J. Biol. Chem. 272 (1997) 24002–24007.
[11] J. MacMicking, Q.W. Xie, C. Nathan, Nitric oxide and macrophage function, Annu. Rev. Immunol. 15 (1997) 323–350.
[12] J.B. Hibbs, R.R. Taintor, Z. Vavrin, E.M. Rachlin, Nitric oxide: a cytotoxic activated macrophage effector molecule, Biochem. Biophys. Res. Commun. 157 (1998) 87–94.
[13] J.B. Hibbs, Infection and nitric oxide, J. Infect. Dis. 185 (Suppl. 1) (2002) S9–S17.
[14] M.W. Cleeter, J.M. Cooper, V.M. Darley-Usmar, S. Moncada, A.H. Schapira, Reversible inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respirator chain, by nitric oxide. Implications for neurodegenerative diseases, FEBS Lett. 345 (1994) 50–54.

[15] G.C. Brown, C.E. Cooper, Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase, FEBS Lett. 356 (1994) 295–298.
[16] G.C. Brown, N. Foxwell, S. Moncada, Transcellular regulation of cell respiration by nitric oxide generated by activated macrophages, FEBS Lett. 439 (1998) 321–324.
[17] N.S. Kwon, D.J. Stuehr, C.F. Nathan, Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide, J. Exp. Med. 174 (1991) 761–767.
[18] M. Lepoivre, J.M. Flaman, P. Bobe, G. Lemaire, Y. Henry, Quenching of the tyrosyl free radical of ribonucleotide reductase by nitric oxide, relationship to cytostasis induced in tumor cells by cytotoxic macrophages, J. Biol. Chem. 269 (1994) 21891–21897.
[19] G. Lemaire, F.J. Alvarez-Pachon, C. Beuneu, M. Lepoivre, J.F. Petit, Differential cytostatic effects of NO donors and NO producing cells, Free Radic. Biol. Med. 26 (1999) 1274–1283.
[20] S. Pervin, R. Singh, G. Chaudhuri, Nitric oxide-induced cytostasis and cell cycle arrest of a human breast cancer cell line (MDA-MB-231): potential role of cyclin D1, Proc. Natl. Acad. Sci. USA 98 (2001) 3583–3588.
[21] G.M. Buga, L.H. Wei, P.M. Bauer, J.M. Fukuto, L.J. Ignarro, N^G-Hydroxy-L-arginine and nitric oxide inhibit Caco-2 tumor cell proliferation by distinct mechanisms, Am. J. Physiol. 275 (1998) 1256–1264.
[22] R.S. Mahidhara, R.A. Hoffman, S. Huang, A. Wolf-Johnston, Y. Vodovotz, R.L. Simmons, T.R. Billiar, Nitric oxide-mediated inhibition of caspase-dependent T lymphocyte proliferation, J. Leukoc. Biol. 74 (2003) 403–411.
[23] E. Raabo, T.C. Terkildsen, On the enzymatic determination of blood glucose, Scand. J. Clin. Lab. Invest. 12 (1960) 402–410.
[24] L.J. Ignarro, G.M. Buga, L.H. Wei, P.M. Bauer, G. Wu, P. del Soldato, Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation, Proc. Natl. Acad. Sci. USA 98 (2001) 4202–4208.
[25] M.R. Kibbe, J. Li, S. Nie, S.C. Watkins, A. Lizonova, I. Kovesdi, R.L. Simmons, T.R. Billiar, E. Tzeng, Inducible nitric oxide synthase (iNOS) expression upregulates p21 and inhibits vascular smooth muscle cell proliferation through p42/44 mitogen-activated protein kinase activation and independent of p53 and cyclic guanosine monophosphate, J. Vasc. Surg. 31 (2000) 1214–1228.
[26] P.M. Bauer, G.M. Buga, L.J. Ignarro, Role of p42/p44 mitogen-activated-protein kinase and p21waf1/cip1 in the regulation of vascular smooth muscle cell proliferation by nitric oxide, Proc. Natl. Acad. Sci. USA 98 (2001) 12802–12807.
[27] A.A. Pieper, A. Verma, J. Zhang, S.H. Snyder, Poly (ADP-ribose) polymerase, nitric oxide and cell death, Trends Pharmacol. Sci. 20 (1999) 171–181.
[28] M. Dalmau, M. Joaquin, T. Nakamura, R. Bartrons, J. Gil, Nitric oxide inhibits DNA synthesis and induces activation of poly(ADP-ribose) polymerase in cultured rat hepatocytes, Exp. Cell Res. 228 (1996) 14–18.
[29] M.P. King, G. Attardi, Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation, Science 246 (1989) 500–513.
[30] B. Beltran, A. Orsi, E. Clementi, S. Moncada, Oxidative stress and S-nitrosylation of proteins in cells, Br. J. Pharmacol. 129 (2000) 953–960.
[31] E. Clementi, G.C. Brown, M. Feelisch, S. Moncada, Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione, Proc. Natl. Acad. Sci. USA 95 (1988) 7631–7636.
[32] A. Bal-Price, G.C. Brown, Nitric oxide induced necrosis and apoptosis in PC12 cells mediated by mitochondria, J. Neurochem. 75 (2000) 1455–1464.
[33] C. Beuneu, R. Auger, M. Loffler, A. Guissani, G. Lemaire, M. Lepoivre, Indirect inhibition of mitochondrial dihydroorotate dehydrogenase activity by nitric oxide, Free Radic. Biol. Med. 28 (2000) 1206–1213.
[34] J.E. Albina, B. Mastrofrancesco, J.S. Reichner, Acyl phosphatase activity of NO-inhibited glyceraldehyde-3-phosphate dehydrogenase (GAPDH): a potential mechanism for uncoupling glycolysis from ATP generation in NO-producing cells, Biochem. J. 341 (1999) 5–9.
[35] E.F. Greiner, M. Guppy, K. Brand, Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production, J. Biol. Chem. 269 (1994) 31484–31490.