

# Progesterone enhances cytokine-stimulated nitric oxide synthase II expression and cell death in human breast cancer cells

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The presence of hormone receptors is related to survival outcome in breast cancer. Previous results from our laboratory established a correlation between the presence of nitric oxide synthase II (*NOSII*) and nitric oxide (NO) production with progesterone receptors in a series of human breast tumours. Furthermore, this was directly related to a lower tumour grade and a lower proliferation rate of the tumour cells. To examine these results in further detail, the effect of progesterone (Pg) and 17 $\beta$ -oestradiol (E2) on *NOSII* expression was analysed in the human breast cancer cell line MCF-7. By Northern blot and promoter activity, we show that a cytokine mix (*TNF- $\alpha$* , *IL- $\beta$* , and *IFN- $\gamma$* ) induces *NOSII* transcription after 6 h stimulation. In the absence of cytokines, neither hormone affects *NOSII* expression. However, Pg but not E2, enhances cytokine-induced *NOSII* transcription as well as NO synthesis, mainly by cooperation with gamma-interferon. The increase in NO accumulation in the media induced by addition of Pg to the cytokine treatment significantly increases cell death, mainly accounted for by apoptosis, as compared to the effect of cytokines alone. Our findings help clarify the role of steroid hormones in *NOSII* expression as well as the effect on cell viability and may suggest novel approaches towards hormone therapy and the treatment of cancer.

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Endogenous nitric oxide (NO) is produced in cells by the nitric oxide synthases (NOS), which exist as three isoforms. The calcium-dependent endothelial (*eNOS* or *NOSIII*) and neuronal (*nNOS* or *NOSI*) enzymes are constitutively present in various cell types and produce low levels of short acting NO.<sup>1</sup> The third isoform (*iNOS* or *NOSII*) is induced in most cell types only in response to external stimuli such as bacterial lipopolysaccharides (LPS) or diverse cytokines, and generates high sustained levels of NO.<sup>2</sup> NO is a transcellular messenger that plays an important role in diverse physiological processes such as vascular homeostasis, immunity and neurotransmission. The effect of endogenous NO on cellular homeostasis is highly dependent on

the level of its synthesis. Thus, at low levels it can promote cell proliferation and angiogenesis whereas at high concentrations, such as those produced by *NOSII*, it can lead to cell damage and death.<sup>3</sup> Therefore, it is important to understand the regulation of the NOS enzymes in order to unravel the role of NO in physiological and pathological conditions. So far it is known that expression of *NOSII* is regulated mainly at the transcriptional level, although additional post-transcriptional and post-translational controls have been documented.<sup>4</sup> The most important transcription factors required for *NOSII* expression are nuclear factor kappa B (*NF $\kappa$ B*)<sup>5</sup> and activator protein 1 (*AP-1*).<sup>6</sup> These transcription factors are induced or activated by cytokines such as tumour necrosis alpha (*TNF- $\alpha$* ), interleukin-1beta (*IL-1 $\beta$* ), gamma interferon (*IFN- $\gamma$* ) or LPS.<sup>7</sup> However, differences in the extent of cytokine-stimulated *NOSII* expression have been observed between diverse cell types.<sup>8</sup> This raises the question of how *NOSII* expression is regulated in different cells and what additional factors contribute to its transcriptional regulation.

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Various studies have reported the presence of all three isoforms of NOS in a variety of tumours of diverse origin. However, the precise effects of NO on tumour growth are complex and remain largely unclear. Several lines of research have indicated that NO may promote tumour growth at low levels,<sup>9</sup> while having a cytotoxic effect at high concentrations that abrogates both tumorigenicity and the formation of metastasis.<sup>10</sup> Thus in human breast cancer, initial studies suggested a higher *NOSII* activity in less differentiated carcinomas, detecting expression of the enzyme predominantly in macrophages. More recently, the level of *NOSII* expression in the cancer cells has been inversely correlated with tumour grade in breast and colon carcinomas,<sup>11–13</sup> and correlated with a higher apoptotic index.<sup>14</sup> This suggests that NO could play a dual role in cancer, depending on the cell type and the concentration of NO produced, a conclusion strengthened by the finding that *NOSII* activity in cells that show increased tumorigenicity is at least an order of magnitude lower than the activity associated with cytotoxicity and apoptosis.<sup>9</sup>

It is well known that the status of steroid hormone receptors is an important prognosis factor in breast cancer.<sup>15,16</sup> Thus oestrogen receptor (*ER*)-negative tumours generally show poorer prognosis than *ER*-positive tumours and the worst scenario arises when both oestrogen and progesterone receptors are absent.<sup>17</sup> These findings led to the establishment of hormone therapy as an important strategy for the treatment of diverse types of cancer, as well as an indicator of the response in breast cancer patients.<sup>18,19</sup> Steroid hormones and their receptors have been correlated with the NO pathway in many systems. Thus, modulation of the constitutive *NOSIII* by hormonal status was suggested in invasive breast carcinomas.<sup>20</sup> Oestradiol has been demonstrated to induce *NOSIII*<sup>21</sup> while inhibiting *NOSII* in different systems.<sup>22</sup> But little is known about the effect of these hormones and their receptors on *NOSII* expression and activity.

We have previously shown that high levels of *NOSII* activity are correlated with lower *in situ* cell proliferation and lower tumour grade in breast carcinoma. We also found a correlation between *NOSII* expression and the presence of progesterone (Pg) receptors.<sup>11</sup> Here we show that Pg potentiates the transcriptional activation of *NOSII* by cytokines, mainly by cooperation with IFN- $\gamma$ . Furthermore, we demonstrate that the expression of *NOSII* and the associated high production of NO induce cell death and that this effect is significantly increased by the presence of Pg, mainly through an increase of apoptosis. Our data help build a molecular explanation for the observed relationship between hormone responsiveness and tumour phenotype in breast cancer.

## Materials and methods

### Cell Culture

Human breast cancer MCF-7 cells were maintained in D-MEM supplemented with 10% foetal calf serum and 2 mM glutamine. The absence of mycoplasma contamination was verified regularly using the Mycotect kit (GIBCO BRL, Cergy Pontoise, France). Before stimulation, cells were depleted of hormones and growth factors in D-MEM without phenol red supplemented with 1% charcoal stripped foetal calf serum (Sigma-Aldrich, France) and glutamine for 24 h (stripped medium). Cells were then stimulated in the same medium.

### Cell Transfection and Reporter Gene Assay

Cells were plated on six-well plates at such a density as to obtain a confluent monolayer ( $1 \times 10^6$  cells per  $\text{cm}^2$ ), and transfected with FuGENE 6 as indicated by the manufacturer (Qiagen Courtaboeuf, France). A pXP2 plasmid containing 7 kb of the *NOSII* promoter, kindly provided by Michael de Vera *et al*,<sup>23</sup> was used to examine transcriptional activity. Cells were routinely cotransfected with a TK-renilla luciferase plasmid, in order to normalise for transfection efficiency. After an overnight incubation, the transfection reagent was removed and fresh controlled medium added. The following day, cells were stimulated with a cytokine mix (CM) of *IL-1 $\beta$*  (3 ng/ml), *TNF- $\alpha$*  (100 ng/ml) and *IFN- $\gamma$*  (300 U/ml) for the appropriate length of time, usually 6 h and/or 17 $\beta$ -oestradiol (E2) (1 nM; Sigma-Aldrich, France) or progesterone (1 nM; Sigma-Aldrich, France). The dual luciferase reporter assay kit from Promega (Madison, WI, USA) was used to determine transcriptional activation following the users manual. In all, 10  $\mu\text{g}$  of total protein were used to perform the dual assays, measuring luciferase and renilla activity in a luminometer Lumat LB9507 (EG&G Berthold, Bad Wilbad, Germany).

### RNA Extraction and Northern Blot

RNA isolation and Northern Blotting was performed as described previously.<sup>24</sup> A 600 bp fragment of the human *NOSII* cDNA (46–644) was used as a probe for *NOSII* detection. This fragment was obtained by enzymatic digestion of a pbluescript plasmid containing human hepatocyte *NOSII* cDNA, kindly donated by Michael de Vera. The sequence was compared with the two constitutive NOS isoforms to verify exclusive homology with *NOSII*. The fragment was purified and labelled using the Promega random primed DNA labelling kit following the corresponding protocol. The labelled probe was purified on a Sephadex 50 column (Boehringer, Mannheim, Germany).

## Electrophoretic Mobility Shift Assay

The assays were performed as described previously.<sup>25</sup> The oligonucleotides were labelled with T4 polynucleotide kinase following the manufacturer's instructions and purified on sephadex 50 columns (Boehringer, Mannheim, Germany) to eliminate unincorporated radioactivity. The consensus *AP-1* and *NFκB* oligonucleotides were obtained from Promega (Madison, WI, USA). The specific antibodies to the *AP-1* family and *NFκB* were purchased from Santa Cruz Biotechnology Inc. (CA, USA).

## Proliferation

Cells were plated in 96-well plates at 10 000 cells/well in complete medium. After a 24-h incubation in depleted medium, they were stimulated as indicated for 24 and 48 h. Following stimulation, the medium was changed adding <sup>3</sup>H-thymidine (1 μCi/ml) and incubating 18 h. After two washes with phosphate-buffered saline (PBS), cells were lysed with 1 M NaOH for 1 h at 50°C and the incorporated radioactivity counted in a liquid scintillation counter (Wallac 1409, EG&G Instruments).

## Cell Mortality and Apoptosis

Cells were treated for the indicated periods of time with either hormone alone or together with cytokines. After stimulation, cells were recovered by trypsinisation and centrifugation, resuspended in phosphate saline buffer. An aliquot was then stained with trypan blue counting the viable (clear) and dead (blue) cells on the light microscope using a cytometer, obtaining the percentages of live and dead cells for each treatment. A second aliquot was used to assess characteristic apoptotic morphological changes. For this purpose, each sample was spread on a glass plate, washed once with phosphate saline buffer and fixed with 3% paraformaldehyde. The nuclei were stained with the Hoechst 33342 reagent (1 mg/ml) for 30 min at room temperature and chromatin condensation was examined using a fluorescent microscope. A total of at least 200 cells in each sample were counted to determine frequency of apoptosis.

## NO Biosynthesis Determination

Cells were plated in six-well plates at  $5 \times 10^5$ /well in complete medium. They were depleted of hormones for 24 h and stimulated for 24 h. The supernatants were recovered and the concentration of NO<sub>2</sub> was determined by the Griess reaction.<sup>26</sup> The NO<sub>3</sub> were reduced to NO<sub>2</sub> by the nitrate reductase and these were measured using the same system.

## Statistical Analysis

The experimental values are expressed as the mean ± standard error of the mean for a number of separate experiments, which is indicated in each case but is in all cases at least three. The Student's *t*-test was used for comparison between two groups or experimental conditions. Significant differences were considered for those probabilities <5% (*P*<0.05).

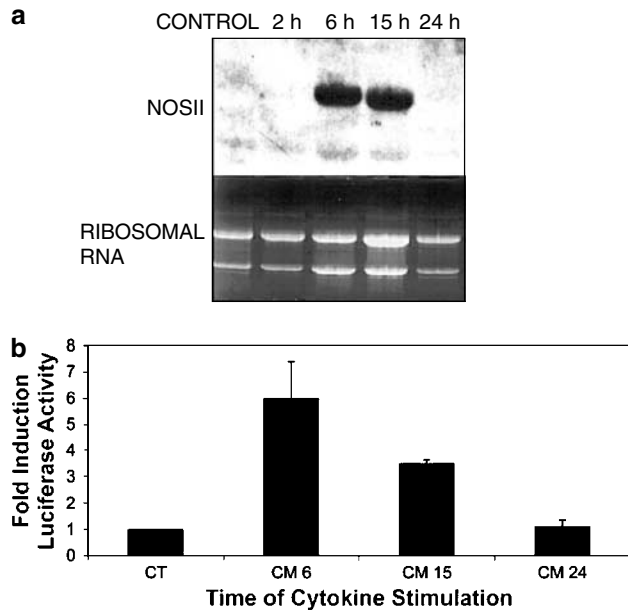
## Results

### NOSII is Induced by Cytokines in MCF-7 Cells

The human breast cancer cell line MCF-7 is a widely used model for the study of human breast cancer. Here, we use this cell line to examine the role of steroid hormones in the expression of *NOSII*. Under control conditions, MCF-7 cells do not express *NOSII*. However, stimulation with a CM containing *TNF-α* (100 ng/ml), *IL-1β* (3 ng/ml) and *IFN-γ* (300 U/ml), induces *NOSII* mRNA accumulation as detected by Northern blotting, which is maximal after 6 h, decreasing thereafter (Figure 1a). These results were confirmed using a luciferase reporter gene containing the proximal 7 kb of the human *NOSII* promoter. The transcriptional activation of the *NOSII* promoter reflects the mRNA accumulation detected by Northern blot being maximal 6 h after cytokine stimulation (Figure 1b). Thus, this is the stimulation time used for the following transcription experiments.

### Progesterone Increases Cytokine-Induced Transcription of NOSII

To study the effect of steroid hormones, it was first necessary to deplete the culture medium of steroids and other compounds such as phenol red that could mimic their activity. Thus, 24 h prior to the experiments, cells were changed into a phenol red-free medium supplemented with 1% charcoal-stripped foetal calf serum. Since culturing breast cells in charcoal-stripped serum has been reported to repress progesterone receptor (*PgR*) expression after 5 days,<sup>27</sup> we performed our experiments within this period and verified by enzyme-linked immunosorbent assay (ELISA) the presence of both steroid receptors in our culture conditions (Table 1). We then examined the effect of steroid hormones on *NOSII* transcription using the reporter gene system described above to measure promoter activity. For this purpose, cells were stimulated with physiological concentrations of Pg (1 nM) and E2 (1 nM) with or without cytokines for 6 h. There is no transcriptional activation in response to either hormone alone, but when added together with CM, Pg increases cytokine-stimulated promoter activity almost two-fold (Figure 2a). This effect is highly significant (*P*<0.001). Consistent with these results,



**Figure 1** Transcription of *NOSII* in response to cytokine stimulation (CM) containing *IL-1 $\beta$* , *IFN- $\gamma$* , *TNF- $\alpha$* . (a) Northern blot for *NOSII*: total RNA was extracted from MCF-7 cells control (CT) or stimulated with CM for the indicated times. The blot was probed for *NOSII* as described in Materials and methods and is a representative result from three independent experiments. (b) Time course of transcriptional activity of *NOSII* promoter: MCF-7 cells were transiently transfected with the luciferase reporter gene containing the human *NOSII* promoter and transfection efficiency was normalised by cotransfection with TK-renilla as described in Materials and methods. Transfected cells were stimulated as indicated, and the results are expressed as fold induction of control nonstimulated cells (means  $\pm$  s.d. of six experiments).

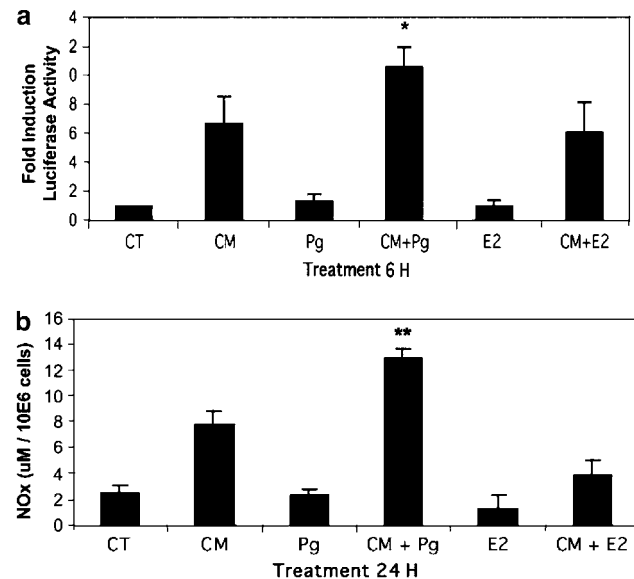
**Table 1** Hormone receptors in MCF-7 cells in the media used

MCF-7	PgR (fM/mg)	s.d.	ER (fM/mg)	s.d.
Standard medium	167.5	9.19	221.5	12.02
Stripped medium	161	8.49	225.5	12.02

Standard medium: D-MEM with 10% foetal calf serum; Stripped medium: D-MEM without phenol red with 1% charcoal-tripped foetal calf serum.

cytokines stimulate NO production, which is further increased by the addition of Pg (Figure 2b). This additive effect of Pg on NO accumulation is significant ( $P < 0.005$ ) and quantitatively consistent with an increase in protein expression. In contrast, E2 shows no effect on cytokine-stimulated *NOSII* promoter activity, on the contrary a decrease in NO accumulation is detected in the media of cells stimulated with CM and E2 (Figure 2a, b).

The effect of Pg on *NOSII* transcription induced by the CM is maximal at a concentration of 1 nM, as determined by a dose-response curve (not shown). Thus, all the following experiments were performed with Pg at this concentration, which is consistent with the physiological levels in breast tissue.



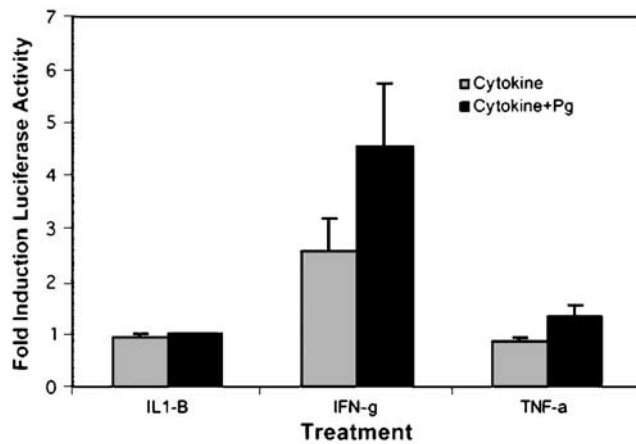
**Figure 2** Effect of steroid hormones on *NOSII* transcriptional activation and NO production. MCF-7 cells were treated as indicated. (a) *NOSII* promoter activity upon treatment with hormones alone or together with cytokines for 6 h: cells were transiently transfected with the reporter gene as described before, and treated as indicated. Luciferase activity is reported as fold induction of nonstimulated cells (means  $\pm$  s.d. of four experiments) \* $P < 0.001$ . (b) NO<sub>x</sub> (NO<sub>2</sub> + NO<sub>3</sub>) accumulation after 24 h stimulation with either hormone with or without cytokines: NO<sub>x</sub> in the media were determined by the Griess method as described. Values represent means  $\pm$  s.d. of four experiments. \*\* $P < 0.005$  vs CM.

We next examined the effect of Pg on each of the cytokines used to stimulate *NOSII* transcription to determine if Pg cooperates with a particular stimulus or adds to the overall stimulation by the mix of the three cytokines used. We and others have shown that the human *NOSII* promoter is poorly stimulated by each cytokine individually, needing the combination of all three for full transcriptional activation. Thus, as expected induction is very low with each cytokine alone, but addition of Pg slightly increases the effect in each case (Figure 3). Most noticeable is the effect on induction by *IFN- $\gamma$*  which is almost doubled by addition of Pg to a level close to that obtained with the combination of the three cytokines, that is, full transcriptional activation.

### Transcription Factors Involved in Hormone Stimulation

In an attempt to unravel the mechanism of the effect of Pg, we studied the effect of the hormone on the transcription factors activated by cytokines that are implicated in the regulation of *NOSII* transcription. These are mainly *NF $\kappa$ B*<sup>5</sup> and *AP-1*,<sup>6</sup> so we examined the effect of both hormones on their DNA binding by electrophoretic mobility shift assay (EMSA). As

expected, the CM induces *NFκB* translocation to the nucleus and the DNA binding of the characteristic p50/p50 and p50/p65 dimers (Figure 4a), but there is no evidence for Pg enhancement of *NFκB* binding. *AP-1* DNA binding is also induced by CM, while neither hormone has any additional effect (Figure 4b). The *AP-1* family of transcription factors identified in MCF-7 cells correspond to *junD*, *c-fos* and a minor contribution of *fosB*, as shown by a supershift with specific antibodies, while the other members of this family, such as *Fra-2* are absent. These results strongly suggest that the Pg-enhancement of cytokine-stimulated *NOSII* expression cannot be caused by a direct interaction of the hormone with the cytokine-dependent transcription factors but imply an independent effect of Pg on promoter activity.



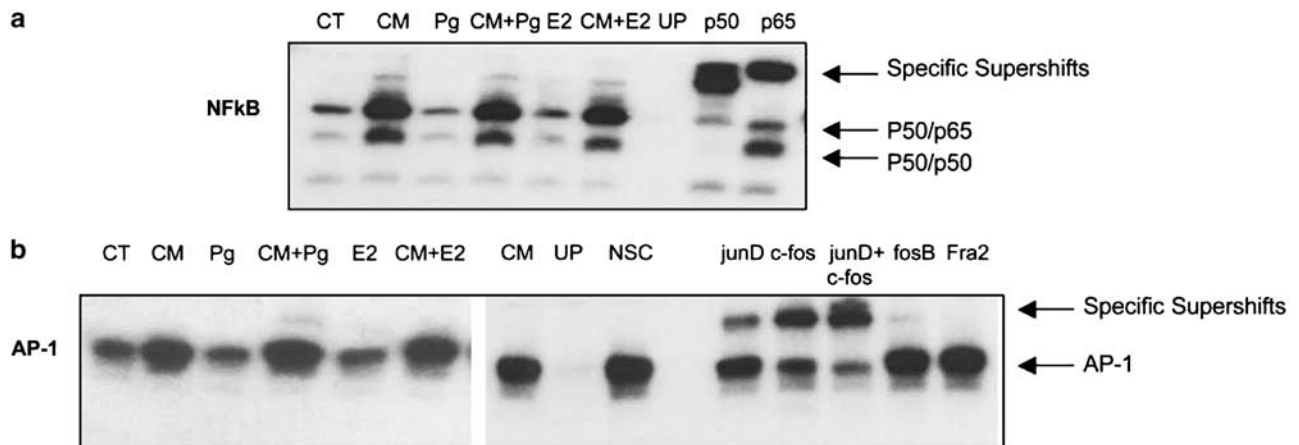
**Figure 3** Effect of Pg on *NOSII* transcriptional activation by each cytokine individually. Cells were transiently transfected with the reporter gene of the *NOSII* promoter and stimulated as indicated for 6 h. The resulting luciferase activity is expressed as fold induction relative to nonstimulated cells.

### Progesterone Increases Cytokine-Induced Cell Death in a NO-Dependent Manner

To place our results into a broader physiological context, we studied both proliferative capacity and cell death in MCF-7 cells after treatment with CM with or without steroid hormones. Proliferation assays show a strong inhibition in the presence of the CM (Figure 5a) that is independent of the further addition of Pg (see Figure 5d). There is no significant effect of Pg alone on cell proliferation. By contrast, as previously reported, E2 stimulates proliferation (Figure 5a). This effect is mediated by the oestrogen receptor since it is inhibited by the known antagonist tamoxifen in a concentration-dependent manner (Figure 5b).

We examined the role of NO in cell proliferation using various NOS inhibitors to interfere with its synthesis. The general NOS inhibitor aminoguanidine (AG) greatly decreases proliferation in the absence of cytokines (Figure 5c), suggesting a role for basal NO production in driving the background rate of cell division. This is probably due to *NOSIII* (*eNOS*), since *NOSII* is not expressed under these circumstances. The role of *NOSIII* in this effect was examined with nitro-L-arginine methyl ester (L-NAME), which selectively inhibits this enzyme without affecting *NOSII*. The results shown in Figure 5c reveal an even stronger inhibitory effect of L-NAME than AG, confirming that basal proliferation as well as the proliferative effect of E2 are mediated by NO synthesised by *NOSIII*.

On the other hand, even though in the presence of CM, proliferation is very low, a slight relief of this inhibition by AG can be seen in Figure 5d. However, these levels of proliferation do not reach the ones observed for untreated cells suggesting the participation of NO-independent mechanisms. Interestingly, AG has no effect on CM + E2, which may be consistent with the results of Figure 2b showing that



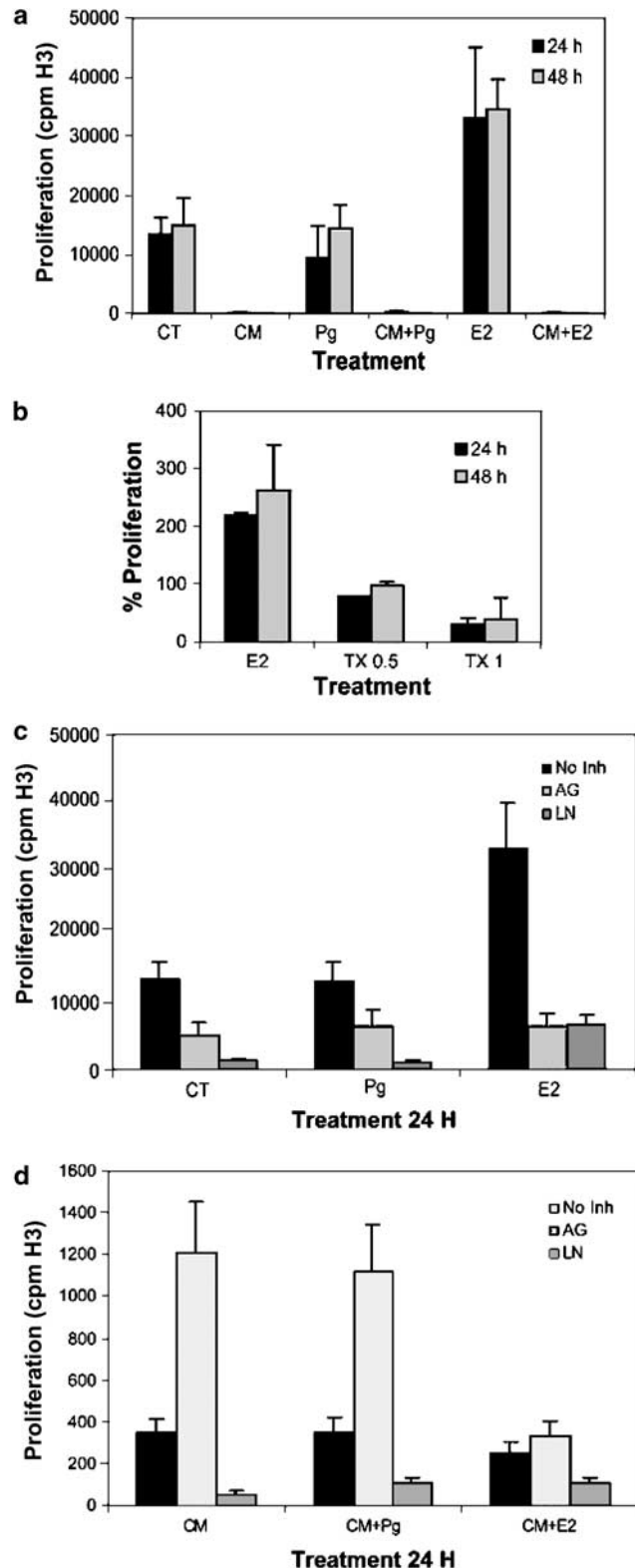
**Figure 4** Effect of hormones on the main transcription factors implicated in *NOSII* transcriptional activation. Cells were treated as indicated for 2 h and EMSAs were performed as described in Materials and methods. (a) nuclear translocation and binding of *NFκB*. The p50 and p65 components of the complex were identified by supershift with specific antibodies. (b) *AP-1* induction in MCF-7 cells and identification of the *AP-1* members in the complexes with specific antibodies. UP: unlabelled probe; NSC: nonspecific competitor.

E2 seems to inhibit NO synthesis induced by cytokines. This agrees with previous reports of *NOSII* inhibition by E2.<sup>22</sup> L-NAME, on the contrary, does not relieve the cytokine inhibition but seems to add to the inhibitory effect, which could be the

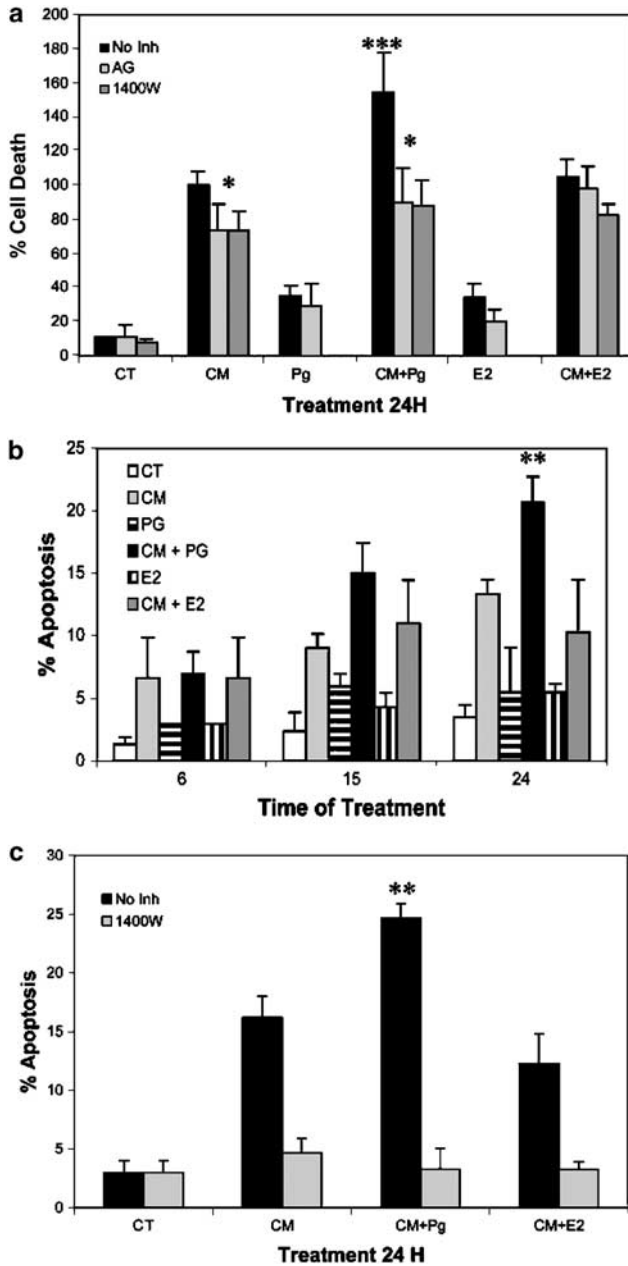
result of inhibiting *NOSIII* and its proliferative effect. Taken together, these results indicate a minor role for *NOSII* in cytokine-induced cytostasis.

Cell death is not significantly increased by treatment with either hormone alone (Figure 6a) and the NOS inhibitor AG shows no effect in these conditions. The cytokine mix induces 30% cell death (adjusted to 100% in Figure 6a) and this effect can be slightly inhibited by AG, suggesting a combination of NO-dependent as well as independent mechanisms. The specific *NOSII* inhibitor 1400W, decreases cell death to the same degree than AG (by 26%), indicating that the high NO output by *NOSII* (Figure 6a) is involved but that other mechanisms play a major role in the general cytotoxic effect of cytokines. The addition of Pg to CM treatment greatly increases the CM-induced cell death (53% increase relative to CM alone, 155% in Figure 6a). This effect is significant ( $P < 0.001$ ) and consistent with the increase of *NOSII* transcription and NO production provoked by the addition of Pg to the CM (Figure 2). The inhibition by AG and 1400W shows that the effect of Pg is mainly NO-dependent and that this NO is produced by *NOSII*, since the level is similar to that obtained by inhibiting the CM treatment. E2 has no effect on cell death neither alone nor when added together with cytokines (Figure 6a).

The general cell death was compared with the frequency of apoptosis by Hoechst staining of treated cells and observation of chromatin condensation on the fluorescent microscope. This staining shows that while neither hormone alone has an effect (Figure 6b), the cytokines provoke apoptosis in a time-dependent manner, reaching 13% after 24 h of treatment. Consistent with the results of cell death, addition of Pg but not E2 causes a significant ( $P < 0.005$ ) increase in the number of apoptotic cells compared to cytokine treatment alone. The effect of Pg is most noticeable after 24 h, when the increase of apoptosis is approximately 35% compared to cytokines alone. The role of *NOSII* and its high NO output in the induction of apoptosis was confirmed by 1400W, which completely abrogates the effect of cytokines with and without either hormone (Figure 6c). These results indicate that Pg is capable of potentiating cytokine induction of *NOSII* expression, thus increasing NO-dependent apoptosis in breast cancer cells.



**Figure 5** Effect of hormones and cytokines on proliferation of MCF-7 cells. Cells were processed and <sup>3</sup>H-thymidine incorporation measured under the various conditions as described in Materials and methods. (a) Proliferation of control (CT) and treated cells measured after 24 and 48 h. (b) Inhibition of proliferative effect of E2 with tamoxifen (0.5 and 1 μM). (c) Inhibition of proliferation by the NOS inhibitor aminoguanidine (AG; 500 μM) and the *NOSIII* inhibitor L-NAME (500 nM) in the absence of cytokines. (d) Effect of the two inhibitors AG and L-NAME on proliferation in the presence of cytokines with or without steroid hormones. All reported values represent means ± s.d. of at least three experiments.



**Figure 6** Cell death of MCF-7 cells upon treatment with hormones with or without cytokines. (a) Mortality revealed by trypan blue staining, after 24 h of the indicated treatment with the general NOS inhibitor aminoguanidine (AG; 500  $\mu$ M) or the specific NOSII inhibitor 1400W (25  $\mu$ M) or without inhibitors (No inh). \*\*\* $P$ <0.001 vs CM; \* $P$ <0.01 vs no inhibitor for both inhibitors used. (b) Time course of apoptosis induced by the different treatments. Apoptotic cells were stained with Hoechst 33342 and counted under the fluorescent microscope. \*\* $P$ <0.005 vs CM. All reported values are means  $\pm$  s.d. of at least three experiments. (c) Effect of 1400W on apoptosis induced by cytokines with or without hormones after 24 h. \*\* $P$ <0.005 vs CM.

## Discussion

Our results show for the first time that Pg potentiates cytokine-induced transcription of NOSII in a human breast cancer cell line. It is well established that full

activation of the human NOSII promoter requires a combination of cytokines, while response to individual stimulation with each cytokine alone is very low.<sup>24,28</sup> Consistent with this mechanism, stimulation with Pg alone might not in itself be strong enough to drive transcription. Moreover, the fact that Pg does not enhance binding of the main transcription factors induced by cytokines, suggests a direct role of the Pg–Pg receptor complex in the activation of the human NOSII promoter. We show that the main cooperative effect of Pg to induce NOSII transcription occurs with IFN- $\gamma$ . This combination achieves the same level of NOSII transcription as the use of the three cytokines together, which may have important implications for therapeutic applications. Previous studies have reported that both Pg and E2 alone induce NOSII expression in different cell types in the uterus<sup>29</sup> and that Pg stimulates NOSII expression and NO production in ovarian cancer cells.<sup>30</sup> In contrast, other studies showed an inhibitory effect of Pg. Thus, Pg inhibited NOSII expression in murine macrophages,<sup>31</sup> as well as in human colon enterocytes DLD-1 and Caco-2.<sup>32</sup> However, it is known that macrophages do not express the Pg receptor.<sup>31</sup> Furthermore, the presence of PgR was not demonstrated in the human enterocytes used in the latter study,<sup>32</sup> but the fact that Pg inhibits NOSII in these cells post-transcriptionally strongly suggests a receptor-independent mechanism since the complex Pg–PgR acts as a transcription factor. It is possible that in the absence of its receptor, Pg acts post-transcriptionally inhibiting NOSII, while when the receptor is present, it quickly sequesters the hormone, translocating it to the nucleus where the complex cooperates with other stimuli to induce NOSII transcription. The conclusion highlights the complexity of the effect of steroid hormones, which varies according to the cell type and its ultimate effect on NOSII expression critically depends on the presence of the receptor.

Treatment of MCF-7 cells with cytokines inhibits proliferation and the fact that this cytostatic effect shows low sensitivity to a NOS inhibitor, implicates alternative pathways. For example, it is known that TNF- $\alpha$  and IFN- $\gamma$  can directly induce G1 arrest by modulating cyclin expression and activation.<sup>33</sup> On the other hand, the inhibition of the receptor-mediated proliferative effect of E2 by AG indicates the participation of a NOS enzyme. Inhibition by L-NAME, a specific inhibitor of NOSIII, and the fact that NOSII is not expressed under these circumstances, indicate that the most likely candidate for this effect is NOSIII. Indeed it has previously been shown that E2 induces NOSIII expression<sup>21</sup> and activity.<sup>34</sup> Several studies have shown a positive correlation of NOSIII and oestrogen receptors in breast tumours<sup>35,36</sup> and that the low level of NO produced by NOSIII is associated with endothelial proliferation.<sup>37</sup> Furthermore, NOSIII is present in human malignant breast tumours where its expression correlates with tumour grade and is associated

with increased breast cancer risk.<sup>38</sup> It is known that the oestrogen-ER complex binds to the p85 $\alpha$  regulatory subunit of PI-3 kinase leading to the activation of *Akt*, and that this pathway is responsible for the phosphorylation of a C-terminal serine of *NOSIII* increasing its catalytic activity. Hence, it is possible that the oestrogen-ER complex can activate the release of NO by *NOSIII* and that this NO may contribute to tumour proliferation and survival.<sup>3</sup>

Beside their cytostatic effect, cytokines are highly cytotoxic for MCF-7 cells. At least two different pathways seem to be involved since AG and 1400W only slightly inhibit cytotoxicity. These pathways may induce diverse types of cell death including apoptosis, necrosis, etc; that will contribute to different degrees to the total cell death. On the one hand, the NO-independent death could be triggered by the cytokines used, such as *TNF- $\alpha$*  via the stimulation of its death domain receptor, while the 1400W/AG-sensitive pathway is consistent with the cytotoxicity caused by high levels of NO produced by *NOSII* in these conditions. These include inhibition of DNA and ATP synthesis, inhibition of cyclin-dependent kinase 2 and accumulation of *p53* (for a review see Xu *et al*<sup>3</sup>). Thus, stimulation of apoptosis due to high NO levels has been described in human ovarian tumour cells.<sup>39</sup> We show here that Pg increases cytokine-induced apoptosis and that the mechanism of this effect is a cooperation with cytokines to increase *NOSII* transcription and NO production. This is confirmed by the observation that both AG and 1400W inhibit the increase in cell death caused by Pg to the same levels as they inhibit the effect of cytokines alone. The reports that Pg induces apoptosis in malignant mesothelioma cells<sup>40</sup> as well as in normal breast cells and various cancer cell lines<sup>41</sup> further support this view. Furthermore, in agreement with the results presented here, the role of Pg in the induction of apoptosis via induction of *NOSII* to increase NO levels was shown in ovarian cancer cells,<sup>30</sup> indicating a general function of Pg in this mechanism. In fact, several randomised studies and clinical trials have shown that treatment with progestogens led to significant though modest improvement of the relapse-free survival,<sup>42,43</sup> and that inclusion of progestogens in hormone replacement therapy reduces the risk of recurrence, which is associated with an increase in the apoptosis/proliferation ratio within the tumour.<sup>44</sup>

The balance between cell death and cell proliferation determines tumour growth rate and even a small alteration in these parameters can be important for the expansion or regression of malignant tumours. Our results show that Pg can increase the inflammatory response against breast cancer cells by augmenting NO levels in the cells, thus leading to apoptosis. We suggest that pharmacological interventions to maximise the Pg-enhanced *NOSII* expression, perhaps in combination with IFN- $\gamma$  may be a plausible option for some forms of breast cancer.

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