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Original Contribution

ADAPTIVE RESISTANCE TO NITRIC OXIDE IN MOTOR NEURONS

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Abstract—Nitric oxide (NO) is a free radical produced actively by mammalian cells, including neurons. Low levels of NO can function in intercellular signaling, but high levels are cytotoxic. This cytotoxic potential suggests that cells at risk for NO damage, such as neurons, might have NO resistance mechanisms to prevent cell death, and adaptive resistance to NO-releasing compounds has been reported for some non-neuronal cell types. Here we show that immortalized mouse motor neurons (NSC34 cells) respond to sub-lethal fluxes of pure NO by activating adaptive resistance mechanisms that counteract cytotoxic NO exposure. This adaptive NO resistance is reversible and is paralleled by the induction of the oxidative stress enzyme heme oxygenase 1 (HO-1). An inhibitor of both HO-1 and heme-dependent guanylate cyclase (tin-protoporphyrin IX) greatly sensitized NO-pretreated NSC34 cells to the NO challenge. However, readdition of cyclic GMP (in the form of the 8-bromo derivative) restored rather little resistance, and a more selective guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxaline-1-one (at 10 μM), did not have the sensitizing effect. Therefore, the inducible HO-1 pathway contributes substantially to adaptive NO resistance, while cyclic GMP seems to play at most a small role. A similar adaptive resistance to NO was observed in primary rat spinal chord motor neurons. The activation of NO resistance in motor neurons may counteract age- or disease-related neurodegeneration. © 1999 Elsevier Science Inc.

Keywords—Free radicals, Heme oxygenase; Oxidative stress; Antioxidants; Gene regulation; Neurodegeneration

INTRODUCTION

Nitric oxide (NO) is a biological free radical with diverse roles. NO is used as a vasodilator that maintains blood pressure [1] and was proposed to act directly on hemoglobin to modulate oxygen transport [2]. Neurons express a neuronal isozyme of NO synthase [3], and neurotransmitter functions for NO have been demonstrated [4]. A separate role for NO as a cytostatic differentiation factor in neurons has also been proposed [5,6]. NO was identified as a key cytotoxic weapon of the immune system, but the relevant inducible form of NO synthase is actually expressed at significant levels in many other cell types [7].

NO toxicity probably occurs through diverse pathways. This potential for broad damage in part reflects the complexity of NO biochemistry. NO combines with oxygen to form deaminating agents, or with superoxide to generate peroxynitrite, a powerful oxidant [8]. Peroxynitrite can damage proteins by forming nitrotyrosine adducts [9], or DNA by forming various lesions [10]. Membranes may also sustain damage from NO [11], so cells have several critical targets for potential NO toxicity.

Cells can respond to free radicals by activating defense responses. *Escherichia coli* bacteria activate the *soxRS* response to resist killing by NO-generating macrophages [12]. Inducible resistance to NO-releasing compounds has been reported, but data addressing the role of NO itself, as opposed to other by-products of decomposition, are mixed [13,14], and such studies for neuronal cells have not been reported. Because NO is implicated in some neurodegenerative diseases [15], the ability of neuronal cells to adjust to varying levels of NO may be physiologically important over their long lifetimes. We have tested the hypothesis that cells encountering pure NO might activate defenses against its toxicity. We find a dramatic adaptive resistance to NO, which involves

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induction of the protective enzyme heme oxygenase 1 (HO-1). Signaling by NO can proceed through heme-containing guanylate cyclase and cyclic GMP (cGMP) production [16], but the induction of HO-1 occurs independently of cGMP, through mRNA stabilization [17]. The inducible resistance to NO in motor neurons appears to correlate with and depend on the induced expression of HO-1.

EXPERIMENTAL PROCEDURES

NSC34 cells were early passage stocks obtained from the N. Cashman laboratory, or in some instances from Dr. A. Sandrock (Harvard Medical School). The cells were grown to 40% confluence in a humidified 5% CO_2 environment in plastic T25 flasks in Dulbecco's modified Eagle's medium (Mediatech; Logan, UT, USA) without sodium pyruvate and supplemented with 10% heat-inactivated, fetal bovine serum. Cell morphology and cell number were determined 48 h after NO challenge. For determining viability, the cells were stained with 0.2% trypan blue and then fixed with 2% glutaraldehyde or methanol vapor [5]. Viable cells were scored as those that remained attached and excluded the dye; none of the non-adherent cells excluded trypan blue. Neurite-bearing cells were scored as those with neurites longer than two cell body lengths [5]. Each data point is the mean of at least four determinations, each representing a count of about 200 cells.

For primary motor neurons from rat spinal cord were isolated from stage E14 Long-Evans rat embryos and maintained in culture essentially as described by Estevez et al. [18]. These cells were plated on flasks coated with a mixture of laminin and poly-D-lysine at a density of 2×10^6 cells per flask and cultured under 5% CO_2 in basal Eagle's medium supplemented with glucose, L-glutamine and 5% fetal bovine serum for 3 days at 37°C. The cells were exposed to NO by the protocol used for NSC34 cells, as described below. After NO exposure, the cells were maintained at 37°C for 48 h, and then fixed with methanol and incubated with Hoechst 33258/bis-benzimide to stain nuclei [19]. The slides were mounted with 0.1 M n-propylgallate in 80% glycerol. Apoptotic cells are identified as those with granular and fragmented nucei and blebbing of the cell membrane, as seen using flourescence microscopy at 600× magnification. Cell viability was assayed as the percentage of cells that remained attached to the flask after NO exposure and the 48-h incubation, and which exhibited normal morphology.

NO gas (highly purified, obtained as 100% NO or as a 10% mixture with 90% argon; Northeast AirGas, Salem, NH, USA), or argon as a control, was passed through gas-permeable Silastic silicon tubing (Dow-Corning) [20] submerged in the growth medium in flasks containing NSC34 cells. The incident NO flux was varied by varying the length of tubing in the medium or the concentration of NO in the gas (10% or 100%). The total NO delivered into solution was determined as nitrite using the Griess reaction [20].

Cells were exposed to the indicated flux of NO delivered over 1 h and incubated an additional 2 h before isolation of total RNA (using the RNeasy kit from Qiagen). Northern blot analysis [21] was performed using mouse HO-1 or β-actin probes, and quantified by scanning densitometry (Millipore Visage system). The graphs show the mean ± standard error of at least 4 experiments.

8-bromo-cGMP was purchased from Calbiochem (San Diego, CA, USA). The HO-1 inhibitor Sn-protoporphyrin IX was obtained from Porphyrin Products (Logan, UT, USA) and used as described [22].

RESULTS

To investigate whether NO can induce neuroprotective functions against oxidative stress in motor neurons, we developed a pretreatment/challenge protocol. To avoid contamination of motor neuron cultures by astrocytes and other cell types, we chose initially to study the NO response of the clonal neuronal cell line NSC34 [23,24]. This hybrid neuron-neuroblastoma cell line possesses the properties of primary motor neurons, including the generation of action potentials, acetylcholine production, the expression of neurofilament triplet proteins, and the innervation of myotubes in vitro [23,24].

NSC34 cells were plated and exposed to subtoxic fluxes of pure NO, incubated to allow induced gene expression, and challenged with a toxic NO flux. Cell death and neurodegeneration were then assessed over a 48-h period after the challenge. In order to control the exposures precisely, we used gas-permeable tubing to introduce pure NO into the culture medium (see Experimental Procedures). This method allowed us to apply continuous, low-level exposures similar to physiological conditions [20]. Humans are estimated to generate >1 mmol of NO per day (for a 70-kg individual), which can be increased 100-fold on immune stimulation [25–27]. These estimates would correspond to average whole-body fluxes of ≥ 0.25 nM/s, with local NO production much higher. By using pure NO, we also avoided the generation of other radical and non-radical by-products produced by NO-releasing compounds [20].

We first established the cytotoxicity of different NO flux rates for the motor neuron cell line. For NSC34 cells under our culture and exposure conditions, a 60-min exposure to NO at ≤ 35 nM/s did not cause detectable cell death or loss of neurites (Fig. 1),

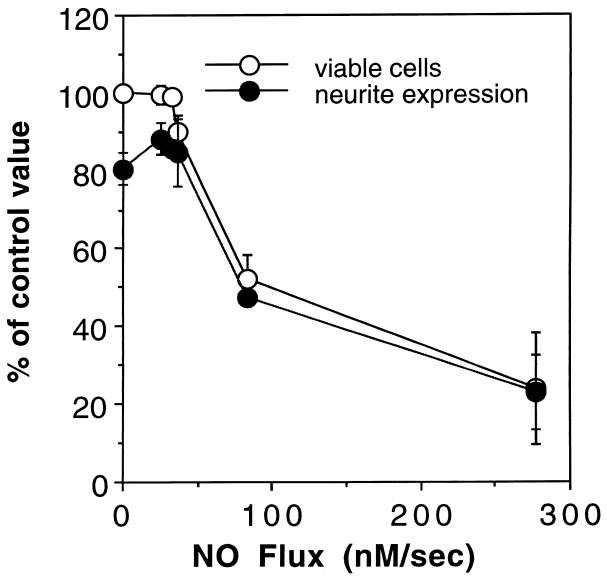

Fig. 1. Viability of NSC34 cells as a function of NO flux. Cells were grown, treated with the indicated NO flux for 60 min, and, after 48 h incubation, analyzed for viability and neurite expression.

a key indicator of neuronal integrity [23,28]. At NO fluxes of 83–277 nM/s, substantial cell death and neurite loss ensued (Fig. 1).

Pretreatment of the murine motor neuron cells for 60 min with a flux of 25 nM/s NO, followed by a 2-h incubation to allow induced gene expression, conferred substantial resistance to a subsequent cytotoxic NO challenge (277 nM/s for 60 min), as judged by cell survival 48 h after the challenge (Fig. 2A–D). The loss of neurite-bearing cells after the NO challenge was significantly less for the pretreated NSC34 cells than for those challenged directly (compare Figs. 2B and 2D). After NO challenge of the non-pretreated cells, microscopic examination revealed the accumulation of considerable cellular debris accompanying degradation and rounding of the remaining adherent cells (Fig. 2B). Among these survivors, the axons of the few neurite-bearing cells were spiny and appeared to be undergoing Wallerian degeneration (Fig. 2B), a morphological characteristic of neuronal degeneration [29].

The time course of NO-induced cytotoxic effects revealed significant protection by the inducing pretreatment as early as 2 h after the challenge (Fig. 3A). For the non-pretreated cells, the loss of trypan blue exclusion at 2 h after the NO challenge was accompanied by cell rounding and shrinkage suggestive of apoptosis (data not shown). A quantitative analysis of neuronal morphology as an indicator of inducible NO resistance showed that essentially all the viable cells in the pretreated population expressed neurites 48 h after the NO challenge, compared to ~35% for the NO-challenged control cells at 48 h (Fig. 3B).

The adaptive resistance to NO probably involves multiple defense pathways, in view of the complex chemistry and array of targets for damage by NO, as noted. We, therefore, tested whether sublethal NO increases resistance to other oxidants. Indeed, after challenge of NO-adapted NSC34 cells with hydrogen peroxide (a 60-min incubation with 100 μM H_2O_2) and subsequent incubation, a vital stain assay showed higher survival in the NO-pretreated cells (77%) than in control cells (40%). Therefore, the adaptive resistance to NO also involves defense mechanisms against H_2O_2.

Induction of resistance to NO was paralleled by the induction of the mRNA encoding the oxidative stress enzyme heme oxygenase 1 (HO-1). As assessed by Northern blotting (Fig. 4A), HO-1 expression was induced rapidly and dramatically in NSC34 cells following subtoxic NO treatment. Treatment with sodium arsenite, a known inducer of HO-1 [17], was used as a positive control (Fig. 4A). When 60-min exposures to various fluxes of NO were followed by a 2-h expression period, HO-1 RNA was induced 5- to 6-fold by 25–40 nM/s NO, but the induction was diminished as the NO treatment entered the cytotoxic range (Fig. 4B). Varying the expression time after a 60-min exposure to NO at 25 nM/s showed that HO-1 mRNA induction was maximal 2 h after treatment, but decreased to control levels by 8 h (Fig. 5A).

HO-1 induction was evidently independent of cGMP: the membrane-permeable analog 8-bromo-cGMP [16] failed to elicit HO-1 mRNA accumulation (Fig. 4A). Moreover, in human fibroblasts, HO-1 induction by NO is not blocked by the inhibitor LY83583 [17]. Thus, the regulatory pathway that governs NO-inducible HO-1 expression involves a novel NO signal transduction mechanism independent of cGMP [17].

The kinetics of adaptive NO resistance in NSC34 cells paralleled the expression of the HO-1 transcript. When the cells were pretreated with a subtoxic NO level and allowed various expression times before a toxic NO challenge, induced resistance was not apparent after only 1 h of expression time following the pretreatment (Figs. 5B and 5C). The maximum resistance was observed at 2 h, which then decayed over the next few hours. The cellular survival reached the control level by 5 h after the pretreatment (Fig. 5B). Neurite expression at this time remained slightly higher (Fig. 5C), but the difference compared to control cells was not statistically significant. These kinetics are consistent with dependence of the induced NO resistance at least in part on HO-1 expression.

To test the role of HO-1 in adaptive NO resistance more directly, NSC34 cells were exposed to subtoxic

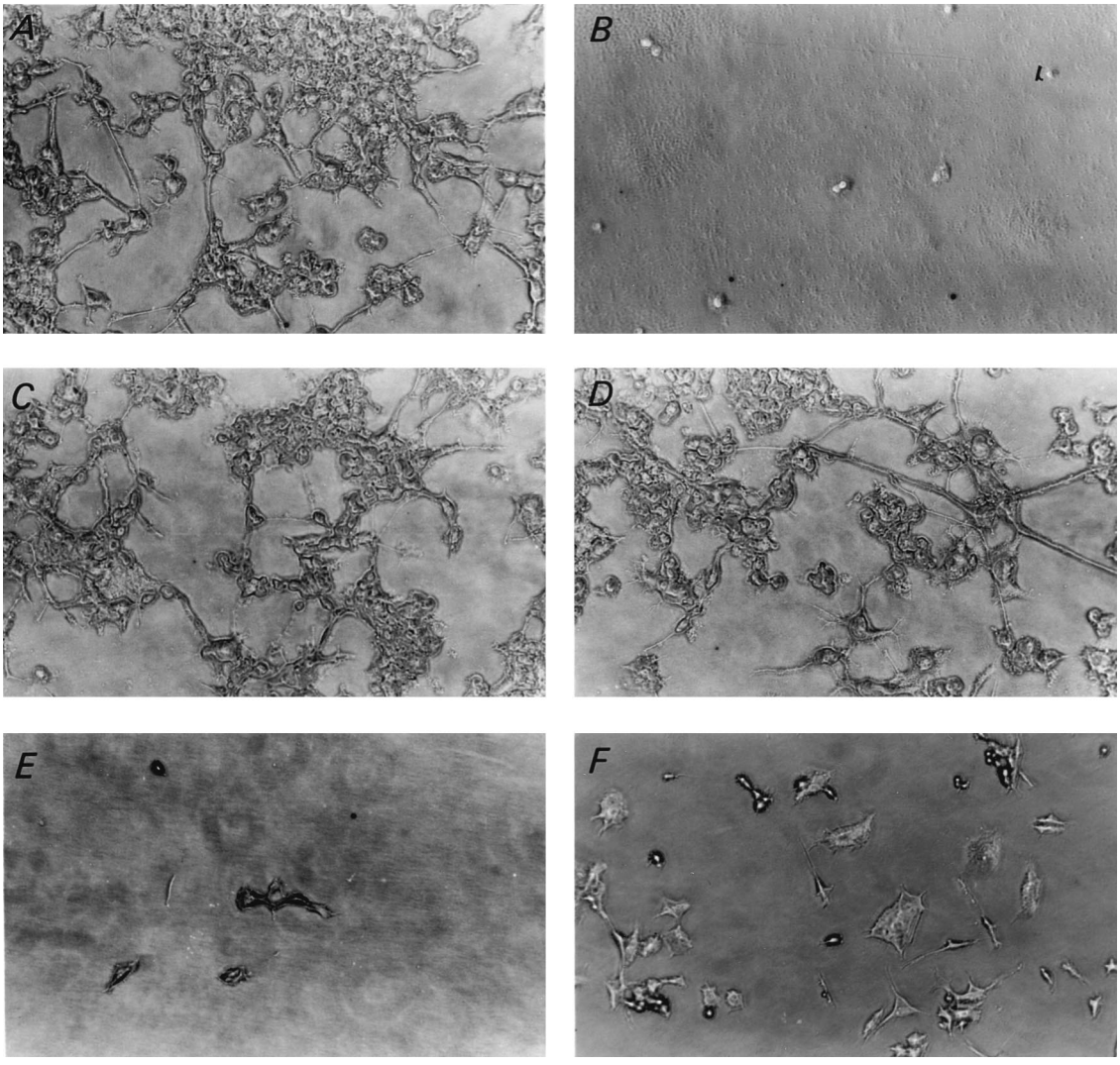

Fig. 2. Viability and neuronal morphology of NO-adapted and challenged NSC34 cells. Cells were pretreated for 60 min with 25 nM/s NO (with added compounds as indicated), incubated 2 h to allow gene expression, and challenged with 277 nM/s NO for 60 min; 48 h after the challenge, the cells were stained with trypan blue, fixed, and analyzed by phase contrast microscopy. A: Non-pretreated, non-challenged cells. B: Non-pretreated cells, challenged with NO. C: NO-pretreated cells, not challenged. D: NO-pretreated cells, challenged with NO. E: Cells pretreated and NO challenged as in (D), with Sn-protoporphyrin (25 μM) present throughout the treatment. F: As in (E), except that 8-bromo-cGMP (20 μM) was also present throughout.

NO in the presence of heme analogs that are HO-1 inhibitors. Because these compounds also inhibit heme-containing guanylate cyclase [16], 8-bromo-cGMP was also added in some experiments in order to restore cGMP pathways. Sn-protoporphyrin did not exert NO-independent cytotoxicity in NSC34 cells (data not shown), but it did abolish the adaptive resistance when present during the NO challenge (Fig. 2E; $\leq$10% survival in three experiments). However, a more selective inhibitor of heme-containing guanylate cyclase (1H-[1,2,4]oxadiazolo [4,3-α] quinoxalin-1-one) did not have this sensitizing effect (data not shown). When 8-bromo-cGMP was added together with the Sn-protoporphyrin inhibitor, adaptive resistance was only partially restored (Fig. 2F; mean survival 27% $\pm$ 3% in three experiments), and incubation of non-pretreated cells with 8-bromo-cGMP did not detectably elevate their resistance to NO (data not shown). Therefore, cGMP-dependent mechanisms contribute little, if at all, to the adaptive NO resistance in motor neurons. Various lines of evidence support an important role for induced HO-1 expression.

Although NSC34 cells exhibit all the cellular and molecular features of motor neurons tested so far [23, 24], it was of interest to determine whether the adaptive NO resistance could be observed in primary motor neurons. For this purpose, cultures of primary motor neurons obtained from rat embryos were tested using the protocol

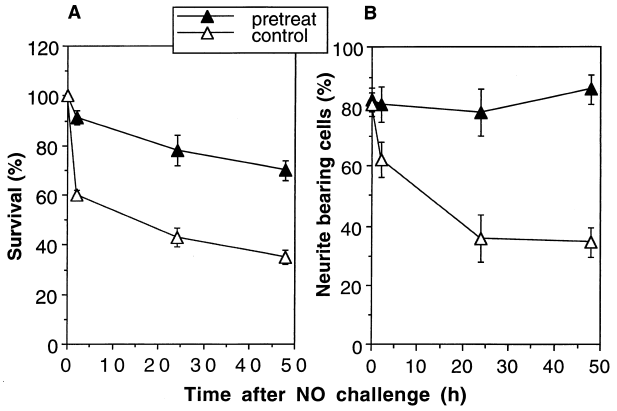

Fig. 3. Quantitation of adaptive resistance to NO. NSC34 cells were pretreated and challenged as described for Fig. 2, then analyzed microscopically at various times after the challenge for viability (A) and neurite expression (B).

developed for NSC34 cells. Microscopic analysis of the cells in this experiment (Fig. 6A–D) showed that the primary cells do indeed have inducible NO resistance activated by the same level of NO exposure as found for the NSC34 cells. Although the degree of induced NO resistance observed for the primary cells (Figs. 6E and 6F) was somewhat lower than that found for NSC34 cells, it seems likely that additional optimization of the pretreatment and challenge levels of NO for the primary cells might produce a more pronounced response. The fundamental conclusion is that adaptive NO resistance is a general feature of motor neurons.

DISCUSSION

Our studies have revealed a dramatic adaptive resistance to a biological free radical whose production may vary greatly due to normal physiological processes. This inducible resistance is triggered by exposure to pure NO, although the biochemical reactions that trigger the response, and many of those that cause cellular damage, remain to be defined. Adaptive NO resistance in NSC34 cells is strongly correlated with the induction of HO-1 (Fig. 5), which seems to constitute a critical component (Fig. 2) of a more complex cellular defense [17]. The induction of HO-1 mRNA has also been reported for non-neuronal cells treated with NO-releasing compounds [13,14,22,30]. However, establishing the role of NO itself is complicated by the by-products of these NO donor compounds; this problem does not apply using the approach [20] we have adopted.

The regulatory mechanisms governing adaptive NO resistance in motor neurons, and their role in neuronal survival, are of great interest. One can find at least two occasions for significant NO exposure of neurons: during

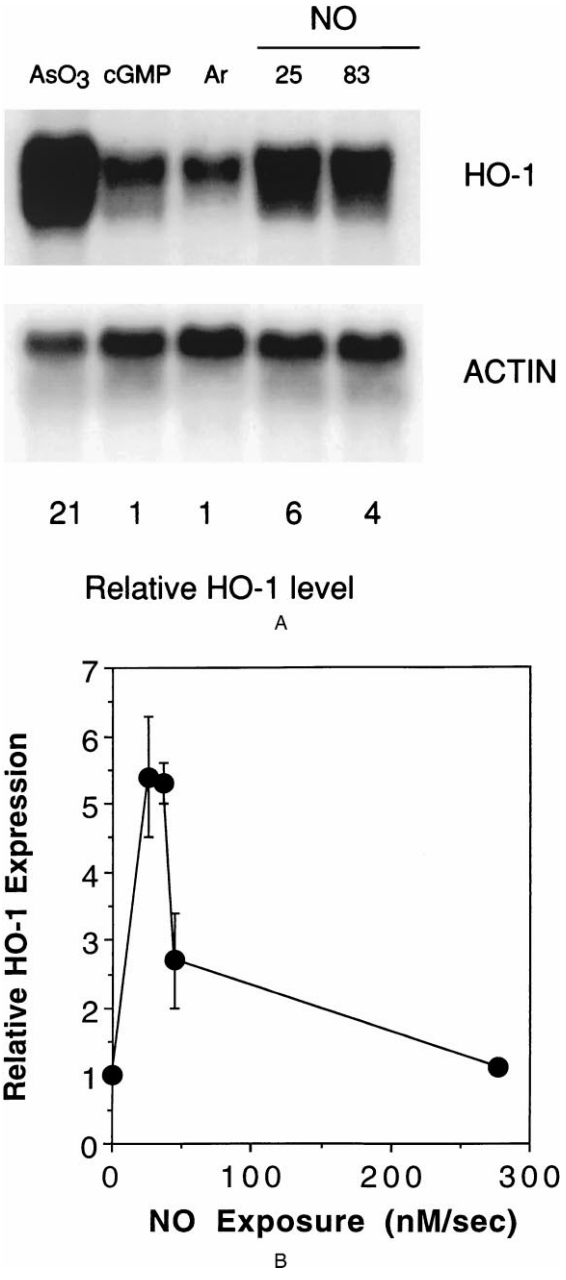

Fig. 4. Induction of HO-1 in NSC34 cells as a function of NO flux. The cells were treated for 60 min with sodium arsenite (AsO_3), 20 μM 8-bromo-cGMP (cGMP) or NO at the indicated flux (in nM/s); Ar indicates control cells treated with argon gas only. Two hours after the treatments, the cells were harvested to isolate total RNA for northern blot analysis. A: Northern blot of HO-1 mRNA (top band) and β-actin (bottom band); the relative HO-1 mRNA level (normalized to β-actin mRNA; the level in argon-treated cells was defined as 1) is indicated below each lane. B: quantitation of HO-1 mRNA induction as a function of NO flux. Each data point is the mean of $\geq$4 independent measurements.

signaling, as found in the hippocampus [31,32], but not yet demonstrated for motor neurons; and during inflammation, when inducible NO synthesis from immune and other cell types transiently generates high local NO con-

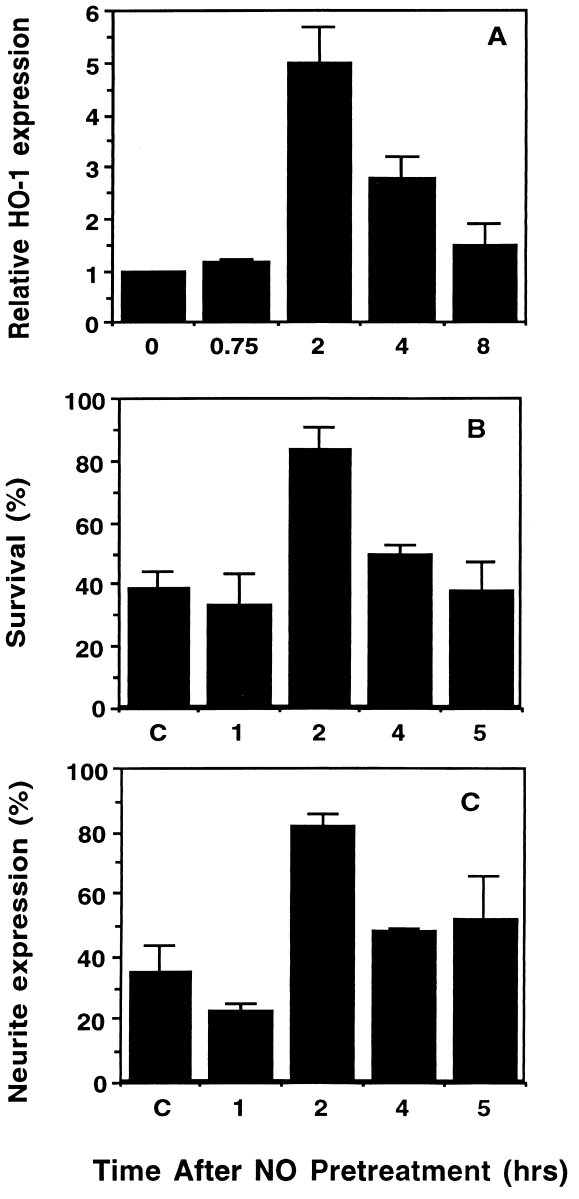

Fig. 5. Kinetics of adaptive NO resistance in NSC34 cells. The cells were pretreated for 60 min with 25 nM/s NO, except for control cells treated with argon gas (labeled "C"). After incubation for the indicated times to allow gene expression, samples were taken for northern blotting (A, showing quantitation as in Fig. 4B), or challenged for 60 min with 277 nM/s NO. After an additional 24-h incubation, viability (B) and neurite expression (C) were measured.

centrations [7,26,33]. Adaptive resistance could certainly occur in the latter case as the NO flux increases over time. The levels of NO that trigger adaptive resistance may be higher than those used for intercellular signaling, but having a such an elevated set-point might avoid triggering the response during normal intercellular signaling.

Cellular damage by NO is also implicated in neurodegeneration accompanying various syndromes, such as amyotrophic lateral sclerosis and stroke [8,34]. HO-1 is up-regulated in the brain tissue of Alzheimer patients relative to control brains [35], which could suggest HO-1 induction by oxidative stress in this disease. Adaptive resistance in neurons may play an important role in limiting or modulating the cellular effects of cytotoxicity in some of these situations.

The specific role of HO-1 in resistance to NO needs to be explored. HO-1 can act in general resistance to oxidants: artificially elevating HO-1 expression confers resistance to hyperoxia in pulmonary epithelial cells [36], and embryonic fibroblasts cultured from HO-1 knockout mice are hypersensitive to various oxidants [37]. In these cases or during adaptive NO resistance, a likely role of HO-1 would involve the production of the antioxidant bilirubin [38]. Alternatively, resistance could arise from secondary regulatory effects of the iron [39] or carbon monoxide [4,16] released during heme degradation. The broad defense functions of HO-1 may be reflected in the elevated H_2O_2 resistance of NO-treated cells, although the HO-1 role needs to be investigated directly.

Our studies of human fibroblasts treated with subtoxic NO levels demonstrate significant induction of at least twelve proteins [17]. This result, together with the increased H_2O_2 resistance of NO-adapted motor neurons noted, points to multiple defense mechanisms operating in adaptive NO resistance. These inducible defenses could prevent or repair damage to DNA, proteins, or the plasma membrane [15,40]. Although the relative importance of cGMP in the adaptive resistance to NO seems small, further tests of individual functions, such as cGMP-dependent protein kinases, is probably warranted [16].

Induction of Mn-containing superoxide dismutase has been reported for a variety of oxidative stress conditions [41], and increased expression of this enzyme could limit the formation of peroxynitrite, a potent derivative of NO and superoxide [8]. The in vivo relevance of peroxynitrite is supported by a study showing that apoptosis in rat motor neurons, induced by the withdrawal of a trophic factor, involves the combined effects of NO and superoxide [18]. Another recent report [42], correlates elevated expression of Mn-containing superoxide dismutase with increased resistance to several NO-donor compounds in PC-12 cells, a non-motor neuron line, and to receptor-mediated toxicity in rat cortical neurons. However, in our experiments, the mRNA encoding Mn-containing superoxide dismutase was not induced in NO-treated human fibroblasts [17] or NSC34 cells (data not shown), which makes it unlikely that this enzyme contributes to adaptive NO resistance as described

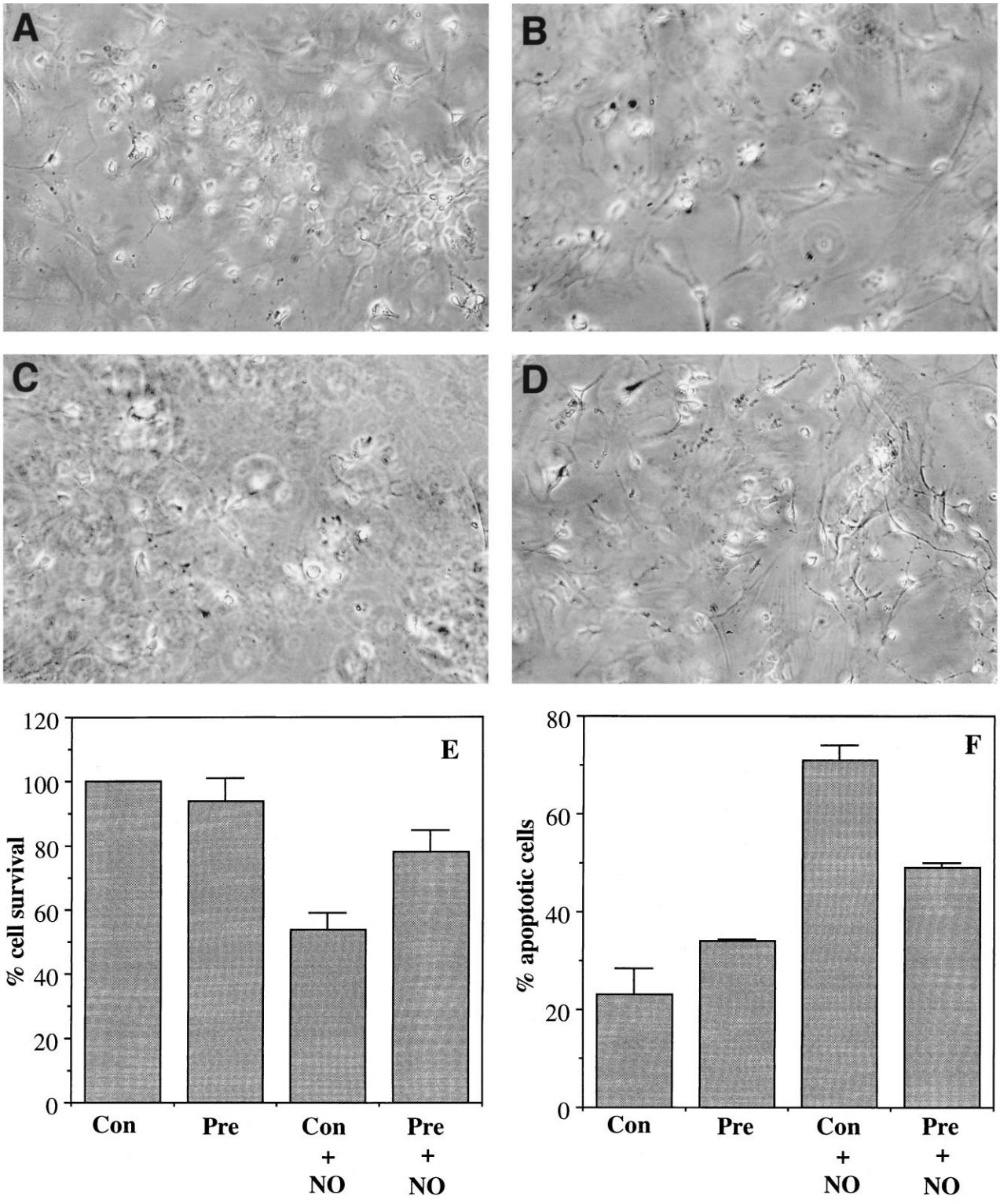

Fig. 6. Adaptive resistance to NO in primary rat motor neurons. Day E14 embryonic motor neurons were isolated (A) and pretreated with 25 nM/s NO for 40 min, followed by and additional 2-h incubation (B). For the NO challenge, the control (C) and pretreated (D) samples were exposed to 125 nM/s NO for 40 min (C and D). After a post-challenge incubation of 48 h, the cells were stained with Hoechst 33258 (shown in the micrographs). Cellular survival (E) and apoptosis (F) was determined by examining fields totaling at least 200 cells. The results in (E) and (F) show the means and standard errors (bars) from two independent experiments using independently isolated motor neuron cultures.

here. Determining the contributions of other activities to adaptive NO resistance ought to shed light on the normal mechanisms of NO resistance in neurons and other cell types, and may illuminate disease processes or the mechanisms of neurodegeneration during aging.

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