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Production of homocysteine in serum-starved apoptotic PC12 cells depends on the activation and modification of Ras

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Abstract

PC12 pheochromocytoma cells expressing a dominant inhibitory mutant of Ha-Ras (M-M17-26) and PC12 cells transfected with normal c-RasH (M-CR3B) have been used to investigate the role of nitrosylation and farnesylation of Ras on the production of homocysteine and the activities of the redox-sensitive transcription factors NF-κB and c-Fos. We found that under serum and nerve growth factor withdrawal conditions undifferentiated apoptotic M-CR3B cells accumulated more homocysteine than M-M17-26 cells, and the production of homocysteine decreased in the presence of manumycin and increased in the presence of L-NAME. Furthermore, we have shown that manumycin increased the activity of c-Fos in the M-CR3B cells and decreased the activity of NF-κB, while L-NAME decreased the activities of both transcription factors, and accelerated apoptosis of M-CR3B cells. In contrast, in M-M17-26 cells manumycin did not change the activity of c-Fos, nor the activity of NF-κB. We conclude that trophic factor withdrawal stimulates Ras, which apparently through the Rac/NADPH oxidase system induces permanent oxidative stress, modulates the activities of NF-κB and c-Fos, induces production of homocysteine and accelerates apoptosis. Nitrosylation of Ras is necessary for maintaining the survival of PC12 cells, while farnesylation of Ras stimulates apoptosis under withdrawal conditions.
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Recent studies have suggested that elevated homocysteine levels are a causal risk factor for Alzheimer's disease [19,28] and Parkinson's disease [14]. Besides, it has been found that patients with severe hyperhomocysteinaemia exhibit a wide range of clinical symptoms, including neurological abnormalities such as mental retardation, cerebral atrophy and seizures [33,34]. Certain drugs used in the treatment of Parkinson's disease also increase the levels of homocysteine and induce an axonal-accentuated degeneration in sensory peripheral nerves [21]. Nevertheless, the molecular mechanisms of homocysteine neurotoxicity, as well as the source of homocysteine in the central nervous system, are not fully understood. Homocysteine induces apoptosis, which is not mediated by NMDA channel-dependent Ca influx, and apparently compromises neuronal homeostasis by multiple, divergent routes [10]. A significant increase in homocysteine was detected following folate deprivation in cultured hippocampal and embryonic cortical neurons, which promotes the accumulation of DNA damage by impairing DNA repair [9,13]. The increasing level of this amino acid was correlated with elevation of reactive oxygen species (ROS), suggesting that oxidative stress may be an important factor for the accumulation of homocysteine in neuronal cells [9].

Homocysteine is a sulfur-containing, nonproteinogenic amino acid biosynthesized from methionine during methyl cycles. It is either catabolized into cystathionine (the transsulfuration pathway) by cystathionine β-synthase, a vitamin B6-dependent enzyme, or remethylated into methionine (the remethylation pathway) by methionine synthase, which uses N^5-methyltetrahydrofolate as methyl donor and cobalamin as cofactor. Cystathionine is then converted into cysteine, a precursor of glutathione, which is the main antioxidant compound of cells. It has been shown that some enzymes of the methyl cycle can be directly affected by oxidative stress. There is some evidence that methionine synthase is inactivated by oxi-

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dation and requires reductive methylation for reactivity, while cystathionine β-synthase is a haem protein and active in the oxidized form [7,31]. These data imply that oxidative stress can diminish remethylation and enhance transsulfuration to maintain, via an adaptive process, the intracellular glutathione pool, whose existence is essential for the redox-regulating capacity of cells.

A large body of evidence suggests that Ras is directly involved in the regulation of the intracellular redox state [2,12,24]. This GTP-binding protein (specifically, Ha-Ras) stimulates intracellular ROS levels by activating NADPH oxidase; inhibition of Ras signalling by farnesylation inhibitors was found to increase resistance to apoptosis in Ha-Ras-expressing endothelial cells [6]. Ras is regulated by a series of post-translational modifications, including farnesylation, methylation, palmitoylation and nitrosylation, but the role of these modifications on the specificity of downstream effectors is not fully understood. Farnesylation is necessary for membrane binding and involves farnesyltransferase-catalysed thioether linkages of farnesyl from farnesylpyrophosphate to C-terminal cysteine residues. The farnesylated Ras is further modified by a specific CAAX protease and methyl transferase and then translocated to the plasma membrane, where it undergoes palmitoylation at multiple upstream cysteine residues (except for K-Ras B). In this regard, *S*-nitrosylation of Ras at Cys-181, Cys-184 or Cys-186 could be important, since it would prevent membrane attachment of Ras or change the palmitoylation/repalmitoylation rate. Structural, chemical and mutational studies have shown that these cysteine residues on Ras are surface-exposed and have the potential to be oxidized by oxidative or nitrosative processes [16,18]. On the other hand, NO-donating reagents stimulated deacylation and permitted subsequent reacylation of Ha-Ras, decreased Ha-Ras GTP binding and inhibited phosphorylation of the kinases ERK1 and ERK2 in NIH 3T3 cells [1]. Thus, nitrosylation altered two important properties of Ha-Ras: membrane attachment and substrate specificity.

In order to clarify these issues, in this study we investigated the effects of farnesyltransferase and nitric oxide synthase (NOS) inhibitors on the synthesis of homocysteine in PC12 pheochromocytoma cells expressing a dominant-negative c-*ras*H gene (M-M17-26), or overexpressing the wild type c-*ras*H gene (M-CR3B).

Cell lines: The M-M17-26 cell line [29] was isolated after transfecting PC12 cells with the cDNA of a dominant-negative c-*ras*H mutant (c-*ras*H-Asn17) under the control of the metallothionein promoter. These cells are resistant to NGF activation of the Ras/ERK pathway and NGF stimulation of neuritogenesis [4]. M-CR3B is a PC12 cell line overexpressing the wild-type c-*ras*H gene from the metallothionein promoter (J. Szeberényi, unpublished results).

Cell culture: M-CR3B and M-M17-26 cells were maintained in DMEM supplemented with 5% fetal bovine serum and 10% heat-inactivated horse serum (Sigma Chemical Co.). Apoptosis was induced by serum withdrawal. For survival experiments, M-CR3B, or M-M17-26, cells were extensively washed with serum-free medium and plated in polylysine-coated 24 well plates at a density of 20×10^4 cells per well.

Cell viability assay: Cell death was estimated by the measurement of lactate dehydrogenase (LDH) [22] released into the extracellular medium by dead or damaged cells after 24 h treatment with L-NAME, manumycin or both. LDH activity was quantified by the NADH oxidation rate, which was followed spectrophotometrically at 340 nm. Total LDH was estimated after lysis of cells in buffer containing Triton X-100 (1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol, 1 mM NaH_2PO_4, 10 mg/ml aprotinin, 10 mg/ml leupeptin and 50 mM HEPES, pH 7.5). The amount of LDH after each treatment, expressed as a percentage of the total LDH, reflects the percentage of dead or damaged cells.

NF-κB and c-Fos DNA-binding activities were determined by BD Mercury Transfactor Kits (Clontech) according to the manufacturer's protocol.

Homocysteine analysis: The quantity of homocysteine was determined by the method of Pfeiffer et al. [25]. Briefly, the samples (50 μL) and 1 μM cysteamine hydrochloride (internal standard) were incubated with 10 μL of 100 g/L tris(2-carboxyethyl)phosphine (TCEP) (Pierce Chemical Co.) for 30 min at room temperature to reduce and release protein-bound thiols, after which 90 μL of 100 g/L trichloroacetic acid containing 1 mM EDTA was added for deproteinization. The sample was centrifuged for 10 min at 13,000 *g*, and 50 μL of the supernatant was added to a vial containing 10 μL of 1.55 M NaOH, 125 μL of 0.125 M borate buffer containing 4 mM EDTA pH 9.5, and 50 μL of 1 g/L 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F, Sigma Chemical Co.) in the borate buffer. The sample was then incubated for 60 min at 60 °C. HPLC was carried out on a solvent delivery system using a fluorescence detector (385 nm excitation, 515 nm emission), both from Waters Technologies Corp. Separation of the SBD-derived thiols was carried out on a NovaPak C18 analytical column (particle size 5 μm, 100 mm × 8 mm i.d., Waters Technologies Corp.) using a 40 μL injection volume and a 0.1 M acetic acid-acetate buffer (pH 5.5) containing 3% methanol as the mobile phase at a flow rate of 0.7 ml/min and a column temperature of 29 °C.

Data analysis: The numerical data from each experiment were analysed separately and treated by one-way analysis of variance (ANOVA). When a significant effect was observed by the ANOVA, Student's *t*-test was also used to compare the samples.

Under growth factor withdrawal conditions, nondifferentiated PC12 cells produce reactive oxygen species [35], which in turn can affect homocysteine metabolism. Under these circumstances, Ras may play an important role since this protein directly regulates the intracellular redox state by activating the Rac/NADPH oxidase systems [17,30]. To investigate the role of nitrosylation and farnesylation of Ras on the viability of cells, homocysteine metabolism and activity of transcription factors, N^{ω}-nitro-L-arginine methyl ester (L-NAME), and manumycin, respectively inhibitors of nitric oxide synthase and farnesyl transferase, were employed. To demonstrate the specificity of Ras-elicited effects, PC12 pheochromocytoma cells expressing a dominant-negative *ras* gene (M-M17-26), and M-CR3B cells

Table 1
Effect of L-NAME and manumycin on extracellular LDH activity

Additions	Extracellular LDH activity (% of total)	
	M-CR3B	M-M17-26
Control	40.4 ± 3.4	45.8 ± 2.4
Control + manumycin (1 μM)	36.5 ± 2.1	50.6 ± 3.6
Control + L-NAME (1 mM)	56.6 ± 4.3*	46.2 ± 3.8
Control + L-NAME (1 mM) + manumycin (1 μM)	41.4 ± 1.2	49.5 ± 2.9

Cells were incubated for 24 h in serum-free DMEM alone or containing L-NAME, manumycin or a combination of L-NAME and manumycin. Extracellular LDH activity is expressed as a percentage of total cellular LDH activity. Values represent the mean ± S.E. of three independent determinations performed in triplicate.
* $P < 0.05$ vs. control.

that had been stably transfected with normal c-*rasH* [29] were incubated with the inhibitors.

We have found that neither manumycin nor L-NAME had an apoptotic effect on M-M17-26 cells at the concentrations used (1 μM and 1 mM, respectively), while L-NAME reduced survival of M-CR3B cells. Under the action of L-NAME, extracellular activity of LDH was increased only in the case of M-CR3B cells (Table 1). These data thus suggest that nitrosylation of Ras is necessary for maintaining the survival of PC12 cells, while farnesylation of Ras stimulates apoptosis under conditions of withdrawal of trophic factors.

Two transcription factors, nuclear factor (NF) kappa B and activator protein (AP)-1, have been identified as being regulated by the intracellular redox state via Ras-dependent pathways [20,27]. Thus, in the next series of experiments the DNA-binding activities of c-Fos and NF-κB were determined. We found that in the M-CR3B cells manumycin increased the activity of c-Fos and decreased the activity of NF-κB, while L-NAME decreased the activities of both transcription factors (Table 2). In contrast to the M-CR3B cells, in the M-M17-26 cells manumycin does not change the activity of c-Fos, nor the activity of NF-κB. Furthermore, in the M-M17-26 cells the effect of L-NAME was of opposite character—it increased the activity of c-Fos. Interestingly, in M-CR3B cells in the presence of both inhibitors the transcription activity of NF-κB was restored to the control level. This effect was not observed in the M-M17-26 cells. Taken all together, these data suggest that nitrosylation and farnesylation of Ras alter the DNA-binding activities of both transcription factors c-Fos and NF-κB.

To test the effects of nitrosylation and farnesylation of Ras and Ras-effector systems on homocysteine metabolism, the levels of homocysteine formed under the action of L-NAME and manumycin were determined. We found that both types of cells produced homocysteine and M-CR3B to a greater extent than M-M17-26 (Fig. 1). The concentration of secreted homocysteine varied within 60–80 nM in the M-CR3B cells and within 40–50 nM in the M-M17-26 cells. In the incubation medium of M-CR3B cells the levels of homocysteine produced statistically significantly decreased under the action of manumycin and increased in the presence of L-NAME. In M-CR3B cells in the presence of both inhibitors the levels of homocysteine were restored to the control level. The effects of manumycin and L-NAME were weaker in M-M17-26 cells. It is interesting to note that an inverse correlation between levels of homocysteine and activity of c-Fos was observed in the MCR3B cells. Such a correlation was not found in the M-M17-26 cells. These findings suggest that nitrosylation and farnesylation of Ras may affect homocysteine metabolism, which finally results in the alteration of the DNA-binding activity of c-Fos.

Oxidative stress appears to be the common convergence condition in the neuronal death process induced by many different insults, including trophic factor withdrawal [23]. Ras is a central molecular switch of intracellular regulation and stress response, and through interactions with downstream effector molecules regulates the activities of several transcription factors [26]. Ras itself is regulated by a series of post-translational modifications, including farnesylation and nitrosylation, but the role of these modifications on stress response and downstream effector specificity is not well understood. Using dominant-negative and wild-type Ras-overexpressing pheochromocytoma cells and trophic factor withdrawal conditions, we have found

Table 2
Enhancer-binding activity of c-Fos and NF-κB after treatment of cells with L-NAME and manumycin

Transcription factors and additions	M-CR3B cells		M-M17-26 cells	
	OD_{450} (mg protein)	Percentage of activity	OD_{450} (mg protein)	Percentage of activity
c-Fos				
Control	0.272 ± 0.021	100	0.187 ± 0.009	100
Control + manumycin (1 μM)	0.327 ± 0.033*	120	0.168 ± 0.014	89
Control + L-NAME (1 mM)	0.211 ± 0.031*	76	0.259 ± 0.021*	138
Control + manumycin (1 μM) + L-NAME (1 mM)	0.226 ± 0.028	83	0.117 ± 0.007*	63
NF-κB				
Control	0.456 ± 0.036	100	0.421 ± 0.027	100
Control + manumycin (1 μM)	0.315 ± 0.027*	69	0.378 ± 0.034	89
Control + L-NAME (1 mM)	0.333 ± 0.031*	73	0.441 ± 0.039	104
Control + manumycin (1 μM) + L-NAME (1 mM)	0.457 ± 0.049	101	0.335 ± 0.033	79

DNA-binding activity of c-Fos and NF-κB was determined after exposure to L-NAME or manumycin for 24 h. All data were normalized to the control values (untreated or only preincubated cells). The data are presented as the mean ± S.E.M. for triplicate determinations.
* $P < 0.05$ vs. control.

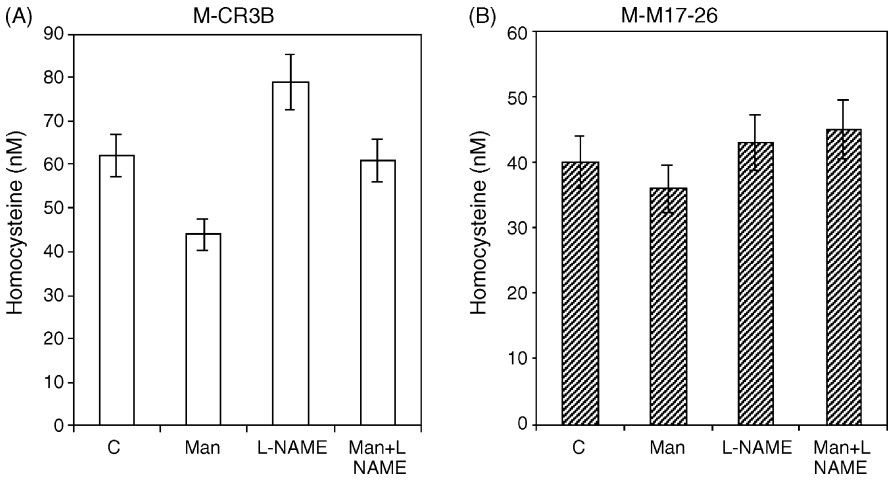

Fig. 1. Production of homocysteine after treatment of M-CR3B and M-M17-26 cells by L-NAME and manumycin. M-CR3B (A) and M-M17-26 (B) cells were cultured for 24 h in the presence or absence of manumycin (1 μM) and L-NAME (1 mM) and aliquots of medium were subjected to HPLC analyses. Homocysteine was not detected in the fresh medium.

that alteration of the steady state levels of nitric oxide and inhibition of farnesylation modifies the activities of c-Fos and NF-κB in the Ras-expressing cells, while in cells with dominant-negative Ras there were no significant effects of these inhibitors.

An increase in homocysteine was detected following folate deprivation in cultured hippocampal and embryonic cortical neurons promoting apoptosis [9,13]. The increasing level of this amino acid was accompanied by the elevation of ROS, suggesting that oxidative stress may be an important factor for the accumulation of homocysteine. Since methionine synthase is inactivated by oxidation, while cystathionine β-synthase is active in the oxidized form [7,31], oxidative stress can reduce remethylation and enhance the transsulfuration that results in increased homocysteine levels. We have determined homocysteine in the cell culture medium after treatment of cells by manumycin and L-NAME and found that addition of L-NAME increased the formation of homocysteine, while manumycin decreased the production of this amino acid in M-CR3B cells. These observations suggest that physiological concentrations of NO appear to regulate metabolic pathways for homocysteine resynthesis by balancing reducing and oxidizing conditions via Ras-operated systems.

We have found an inverse correlation between levels of secreted homocysteine and the DNA-binding activity of c-Fos. Reduction of the activity of c-Fos in M-CR3B cells was accompanied by an increasing level of homocysteine, and conversely elevated activity of c-Fos correlated with diminution of homocysteine production. Homocysteine might use several distinct mechanisms to affect signalling processes leading to stimulation of gene expression in PC12 cells. As an inhibitor of protein methylation, it was found to inhibit NGF-induced neurite formation, supposedly through blocking the methylation of key signalling proteins [5]. In contrast, homocysteine treatment of cortical neurons was found to evoke increased phosphorylation of the tau protein via the ERK pathway, a signalling route that stimulates the expression and phosphorylation of Fos, and the enhancer-binding activity of the AP-1 complex [10]. Our results correlate with the first observation: an increase in homocysteine concentration interferes with c-Fos DNA-binding in PC12 cells. On the other hand, a positive correlation between secreted homocysteine and the viability of M-CR3B cells was also found. This correlation was observed only in the Ras-overexpressing M-CR3B cells, whereas in the Ras dominant-negative M-M17-26 cells such dependence was not found. Besides, we have shown that in the M-CR3B cells manumycin increased the activity of c-Fos and decreased the content of homocysteine. All together, these observations suggest that homocysteine is accumulated during trophic factor withdrawal conditions via Ras-dependent oxidative stress, and an elevated concentration of this amino acid can depress the DNA-binding activity of c-Fos. Taking into account that mitogenic stimuli preferentially recruit the ERKs and activate AP-1 through expression enhancement of AP-1 components [11,15], while NGF withdrawal leads to sustained activation of the JNK and p38 enzymes and inhibition of ERKs in PC12 pheochromocytoma cells [35], we infer that depression of the activity of c-Fos in our experiments reflects rearrangements of subunits in the AP-1 system.

Our results show that depression of the concentration of NO by L-NAME accelerates apoptosis of M-CR3B cells, but this inhibitor does not change the viability of M-M17-26 cells. Furthermore, we found that L-NAME decreased the activity of c-Fos in the M-CR3B cells and increased the production of homocysteine. Since inhibition of NOS by L-NAME changes the viability of the M-CR3B cells only, we may conclude that diminishing the content of NO directly affects Ras, and that this action is not mediated by the nitrosylation of another regulatory protein, e.g. c-Fos [8]. Our results agree with the observation of Teng et al. [32] that activation of c-Ha-Ras by NO modulates survival responsiveness in PC12 cells and can protect them from apoptosis.

In the presence of manumycin the activity of NF-κB in M-CR3B cells was decreased, indicating that farnesylation of Ras is necessary for the activation of this transcription factor. Further-

more, we found that diminution of the intracellular concentration of NO by L-NAME decreased the activity of NF-κB, but paradoxically in the presence of both manumycin and L-NAME the activity of NF-κB was restored to the normal level. These data suggest that both nonfarnesylated and nonnitrosylated Ras act on the same downstream effectors as does doubly modified Ras. Our observation agrees with the suggestion of Bergo et al. [3] that the absence of carboxyl methylation of CAAX proteins has prominent effects on Ras turnover, but apparently little or no effect on downstream effector specificity.

Farnesylated Ras is modified by a specific CAAX protease and is translocated to the plasma membrane where it undergoes palmitoylation at multiple upstream cysteine residues. Under this circumstance, *S*-nitrosylation of terminal cysteines of Ras could be important, since it would prevent membrane attachment of Ras. Structural, chemical and mutational studies have shown that these cysteine residues of Ras are surface-exposed and have the potential to be oxidized by oxidative or nitrosative processes [16,18]. Cys-186 is stably modified with isoprenoid in the mature Ha-Ras protein and is available as a nitrosylation target only in the unmodified precursor form. If exposure to nitric oxide or farnesyl transferase inhibitors were to cause accumulation in this usually minor population, then *S*-nitroso modification of Cys-186 could assume a larger importance. However, further study is needed for clarification of the substrate specificities of nitrosylated and farnesylated Ras.

In conclusion, our results indicate that trophic factor withdrawal stimulates Ras (apparently Ha-Ras), which probably through the Rac/NADPH oxidase system induces permanent oxidative stress. The elevation of reactive oxygen species causes inhibition of methionine synthase and increases the concentration of homocysteine, which in turn accelerates cell apoptosis. Under these circumstances the DNA-binding activities of two redox-sensitive transcription factors, NF-κB and c-Fos, were altered, modulations which depend on the levels of nitrosylation and farnesylation of Ras.

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