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Nitric oxide mediates either proliferation or cell death in cardiomyocytes. Involvement of polyamines

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Summary. Nitric oxide (NO) is a molecule involved in several signal transduction pathways leading either to proliferation or to cell death. Induction of ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis, represents an early event preceding DNA synthesis. In some cell types increased ODC activity seems to be involved in cytotoxic response. We investigated the role of NO and ODC induction on the events linked to cell proliferation or to cell death in cultured chick embryo cardiomyocytes. Exposure of cardiomyocytes to tumor necrosis factor (TNF) and lipopolysaccharide (LPS) caused NO synthase (NOS) and ODC induction as well as increased incorporation of [^{3}H]-thymidine. This last effect was blocked by a NOS inhibitor and was strongly reduced by difluoromethylornithine (DFMO), an irreversible inhibitor of ODC. Sodium nitroprusside (SNP), an exogenous NO donor, inhibited the increases of NOS and ODC activities and abolished the mitogenic effect of TNF and LPS. Moreover, SNP alone caused cell death in a dose dependent manner. The cytotoxicity of SNP was not affected by DFMO while it was prevented by antioxidants. The results suggest that different pathways would mediate the response of cardiomyocytes to NO: they can lead either to ODC induction and DNA synthesis when NO is formed through NOS induction or to growth inhibition and cell death, when NO is supplied as NO donor. Increased polyamine biosynthesis would mediate the proliferative response of NO, while the cytotoxicity of exogenous NO seems to involve some oxidative reactions and to depend on the balance between NO availability and cellular redox mechanisms.

Keywords: Amino acids – Nitric oxide – Ornithine decarboxylase – Proliferation – Cell death – Cardiomyocytes

Introduction

Nitric oxide (NO) is a chemical messenger relevant for cardiovascular pathophysiology (Dinerman et al., 1993). NO is synthetized from L-arginine by

a family of NO synthases (NOS), whose genes may be consitutively expressed and/or induced by several stimuli through transcriptional activation in different cell types, including cardiomyocytes (Schulz et al., 1992; Knowles and Moncada, 1994). In the cardiovascular system NO, produced by inducible NOS, mediates the negative inotropic effect of cytokines (Finkel et al., 1992). Moreover, in adult cardiomyocytes, NO generated by NOS induction can be potentially cardiotoxic (Pinsky et al., 1995). Stimulation of NOS by cytokines induced apoptosis in macrophages (Sarih et al., 1993) and murine mastocytoma (Kitajima et al., 1994). The cytokine tumor necrosis factor (TNF) either stimulated proliferation or induced apoptosis depending on the cell type and/or the developmental stage (Hurme, 1988; Golstein et al., 1991). The exogenous NO donor sodium nitroprusside (SNP), either promoted DNA synthesis (Ziche et al., 1994) or inhibited cell proliferation (Yang et al., 1994) in endothelial cells. Exogenous NO induced apoptosis in chondrocytes (Blanco et al., 1995) and in aortic smooth muscle cells (Nishio et al., 1996), where NO donors act through a cGMP-independent mechanism. In other cell types NO donors can protect from cytotoxicity and prevent apoptosis (Polte et al., 1997; Ogura et al., 1997). Induction of ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis, represents an early event preceding DNA synthesis (Tabor and Tabor, 1984). In some cells treated with TNF, the increases of ODC activity and of polyamine content, together with an higher expression of *c-fos* and *c-myc*, represent intracellular signals leading to enhanced [^{3}H]–thymidine incorporation (Manchester et al., 1993). In other cells, increased ODC activity and *c-myc* expression are implicated in the cytotoxic response leading to cell death (Askew et al., 1991). In cells possessing arginase, the ornithine required for polyamine synthesis may be derived from arginine, which is also a precursor of NO. Therefore, treatment of the cells with inhibitors of ODC might be expected to increase the availability of arginine for NO synthesis. However, in macrophages the selective inhibitor of ODC, α-difluoromethylornitine (DFMO) results to decrease NO production by lipopolysaccharide (Morgan, 1994). A negative correlation between polyamines and NO generation has been reported in rat cerebellum (Hu et al., 1994).

In neonatal rat cardiomyocytes the increase of ODC activity and of polyamine content results to be involved in hypertrophy (Toraason et al., 1990). It is becoming evident that pathological hypertrophy frequently appears in the same context of cell death and it has been suggested that apoptosis may be an endpoint for the hypertrophic process. Recently it has been observed that cardiac hypertrophy is also accompanied by enhanced production of factors, such as the atrial natriuretic peptide (ANP) which, in neonatal rat cardiac myocytes, inhibits cardiac growth and induces apoptosis in a dose-dependent manner (Wu et al., 1997). In adult cardiomyocytes NO produced by cytokines is though to cause apoptosis through cGMP generation (Pinsky et al., 1995), similar to ANP. Interestingly, in cardiac myocytes angiotensin II as well as hypoxia activate both growth and death stimulating pathways, even if it has been suggested that pathways regulating cell growth and apoptosis are in fact divergent and the precise point of divergence is under investigation (Bishopric et al., 1997).

Recently it has also been shown that cardiomyocytes can undergo apoptosis following ischemia and reperfusion, possibly through a mechanism of DNA damage by free radicals (Umansky et al., 1995) and that oxidative DNA damage can be induced by NO donors (Inoue and Kawanishi, 1995).

In the present work we studied the relationship between NO and ODC induction in events linked to cell proliferation or cell death in cultured chick embryo cardiomyocytes. We showed that different pathways, which cause ODC induction, would mediate the response of heart cell to NO. They can lead either to DNA synthesis, when NO is formed through NOS induction, or to growth inhibition and cell death when it is supplied as NO donor. We also show that ODC induction is required for stimulation of DNA synthesis while it would not be implicated in the cytotoxic effect of NO. This last effect appears to involve a reaction of NO with reactive oxygen species.

Materials and methods

Preparation of cardiomyocyte cultures

Cardiomyocyte-enriched cultures were prepared from the hearts of 10 day-old chick embryos by a trypsin disaggregation procedure (Pignatti et al., 1990). To decrease non-myocyte contamination, dissociated cells were preplated for 2 h at 37°C, after which unattached cells (cardiomyocytes) were resuspended in DMEM (GIBCO) supplemented with 10% foetal calf serum, 1% streptomycin, 1% penicillin, seeded in 60 mm dish at a density of 3×10^6 cells and grown to confluency at 37°C in a humidified atmosphere containing 5% CO_2. Confluent cultures, maintained for 20 h in a serum-free DMEM, were then treated with the different drugs as described in the legends. All the reagents were dissolved in serum-free DMEM except for vitamin E and Trolox which were dissolved in ethanol and then diluted in the medium. The final ethanol concentration was kept constant at 0.2% and did not affect the parameters under investigation.

Nitric oxide synthase assay

NOS activity was tested monitoring L-[^{3}H]-citrulline formation from L-[2,3-^{3}H]-arginine. At the end of the incubation periods, the cells were washed once with Hepes buffer and then incubated for 30 min at 37°C with 1 ml of the same buffer containing 10 mM L-arginine and 1 μCi L-[2,3-^{3}H]-arginine (NEN, 40.5 Ci/mmol specific activity)/plate. The reaction was stopped by washing the cells with cold phosphate buffered saline (PBS) containing 5 mM L-arginine and 4 mM EDTA. After supernatant removal, 0.5 ml ethanol was added to each monolayer and allowed to evaporate. Two ml of 20 mM HEPES, pH 5.5 were then added. After 5 min, 1 ml of supernatant was mixed with 0.4 ml of blurry Dowex AG50W-X8 Na+ form equilibrated in stop buffer and vortexed for 30 min. Thus, 0.5 ml were collected from the supernatant and counted in a Canberra Packard MINAXI tri-carb 4,000 series liquid scintillation spectrometer.

Ornithine decarboxylase assay

At the end of the incubations a crude enzyme extract was prepared from cells which were previously washed with PBS and scraped in a buffer consisting of 0.1 mM EDTA, 0.02 mM piridoxal phosphate, 2.5 mM dithiothreitol in 10 mM sodium phosphate buffer, pH 7.2. The cells were distrupted by freeze-thawing three times and then centrifuged at

11,000 rpm for 15 min. The enzyme activity was measured by estimation of the release of $^{14}CO_2$ from L-[1-^{14}C]-ornithine. Briefly, 50 μl of the supernatant was reached with 0.05 μCi of L-[1-^{14}C]-ornithine (58 mCi/mmol) and 6 nmoles of unlabelled ornithine. The reactions were performed in plastic tubes each fitted with a paper disc impregnated with 20 μl of protosol and transfixed to a disposable syringe needle which pierced the plastic cap. After 1 h of incubation at 37°C, the reaction was terminated by injecting 0.1 ml of 10% TCA through the syringe needle, which was then stoppered and the incubation was continued for additional 30 min to ensure complete release of radioactive CO_2. The paper discs were then assayed in a liquid scintillation counter. Data are expressed as pmol/mg protein/h. Proteins were determined according to Bradford (1976).

[^{3}H]-Thymidine incorporation

DNA synthesis was quantified by [^{3}H]-thymidine incorporation of subconfluent cardiomyocyte cultures. The cells maintained for 20 h in a serum-free DMEM and then treated with the different drugs, were pulsed during the last 2 h with 3 μCi of [^{3}H]-thymidine per dish (Amersham, 5.0 Ci/mmol specific activity). The cells were then washed twice with ice-cold PBS, collected by scraping in cold 0.6 M perchloric acid, frozen and thawed twice and centrifuged at 15,000 g for 10 min. The precipitate, dissolved in 1N NaOH, was used for radioactivity analysis. Data are expressed as % of the radioactivity measured under basal condition.

Cell death

Quantitative assay of cell death was performed by measuring lactate dehydrogenase (LDH) leakage into the medium from damaged cells (Van Heugten et al., 1994). For this purpose, the fraction of LDH activity released from dead cells was measured spectrophotometrically in the medium. To obtain total LDH activity, cardiomyocytes from other plates, treated in the same manner, were collected by scraping in the medium, frozen and thawed twice and then centrifuged. The percent of LDH released represents the fraction of LDH activity found in the medium, with respect to the overall enzyme activity.

Data are the means ± SD of 6 determinations from 3 different experiments.

Results

Pro-inflammatory cytokines are known to stimulate inducible NOS activity in different cultured cell types (Nathan, 1992). As reported with adult mammalian heart myocytes (Stein et al., 1996), the treatment of confluent and serum-starved chick embryo cardiomyocytes with tumor necrosis factor-α (TNF) (500 U/ml) and E. Coli lipolysaccharide (LPS) (10 μg/ml) induced NOS activity (Fig. 1A). SNP (10 μM), an exogenous NO donor, administered 30 min before and during TNF and LPS challenge, completely prevented the induction of NOS (Fig. 1A). The ability of exogenous NO to modulate its own synthetic machinery, by affecting inducible NO synthase mRNA expression, was also observed in microglial cells (Colasanti et al., 1995) and in adult rat cardiomyocytes (Giordano et al., 1996). Besides, the addition of TNF and LPS to cardiomyocyte cultures caused the induction of ODC, key enzyme of polyamine biosynthesis and universal marker of cell proliferation (Fig. 1B). Again, pretreatment with 10 μM SNP strongly reduced ODC induction by TNF and LPS. SNP alone, at this dose, did not affect basal NOS and ODC

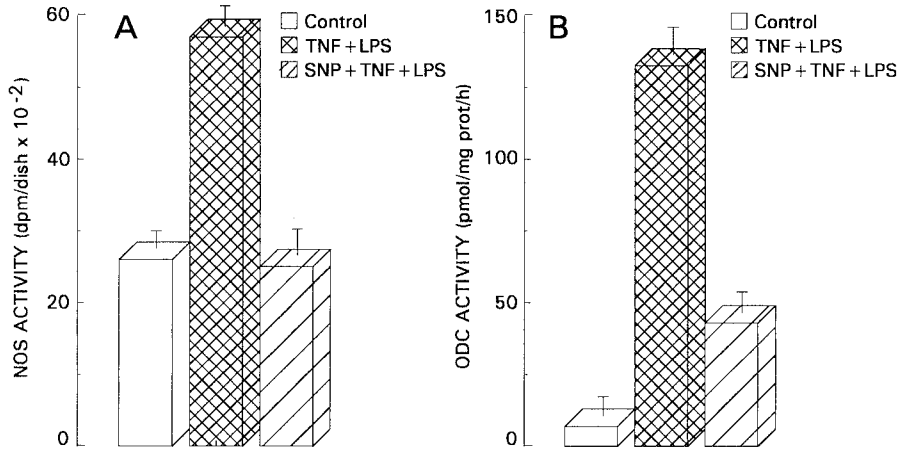

Fig. 1. TNF+LPS induce NO synthase (**A**) and ODC (**B**) activity in cultured cardiomyocytes. Confluent and serum starved cardiomyocytes were incubated for 4 h with serum-free DMEM in the absence (Control) or presence of 500 U/ml TNF+10 μg/ml LPS. 10 μM SNP was added to the cultures 30 min before TNF+LPS. Data are expressed as dpm/dish (*NOS*) and pmol CO_2/mg prot/h (*ODC*). Values are the mean ± SD of triplicate experiments

activities nor cell viability up to 8 h of incubation (not shown). Induction of ODC and NOS by TNF and LPS might not be linked by any cause-effect relationship since neither pretreatment with DFMO, an irreversible inhibitor of ODC, nor pretreatment with the NOS inhibitor L-N-monomethylarginine (NMMA) did affect NOS or ODC activity, respectively (not shown). Moreover, the treatment of confluent and serum starved cardiomyocytes with TNF and LPS caused DNA synthesis after 20 h, as revealed by enhanced [^{3}H]-thymidine incorporation into the acid insoluble fraction (Fig. 2). Although we cannot rule out other targets for TNF and LPS, both NOS and ODC inductions appear to be necessary steps, since preincubation with NMMA or DFMO prevented the mitogenic effect of TNF and LPS. As observed for NOS and ODC induction, pretreatment with SNP also abolished the effect of TNF and LPS on DNA synthesis (Fig. 2). Moreover, prolonged (20 h) exposure of cardiomyocytes to SNP alone caused cell death in a dose-dependent manner (1–100 μM), as indicated by LDH release into the culture medium (Fig. 3). Conversely, TNF and LPS treatment did not lead to cytotoxicity (Fig. 3). The cytotoxicity of 100 μM SNP was not dependent on polyamine biosynthesis, slightly increased by this dose of SNP (not shown), since the release of LDH by SNP was not changed in DFMO-pretreated cardiomyocytes (Fig. 4). On the contrary, the presence of antioxidants such as Vitamin E, Trolox, a water soluble analog of vitamin E, or N-acetylcysteine counteracted the release of LDH stimulated by SNP (Fig. 4), supporting for an involvement of oxidative mechanisms in the cytotoxic action of SNP. Data not shown indicated that in chick embryo cardiomyocytes necrosis and not apoptosis could be the mechanism of cell death by SNP since, contrary to that observed in the presence of

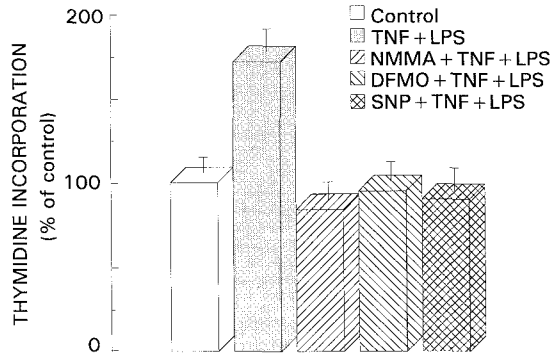

Fig. 2. TNF+LPS stimulate DNA synthesis in cultured cardiomyocytes. Confluent and serum-starved cardiomyocytes were incubated for 20 h with 500 U/ml TNF+10 μg/ml LPS in the absence (Control) or presence of 4 mM DFMO, 100 μM NMMA or 10 μM SNP. NMMA or SNP were added to the cultures 1 h or 30 min before TNF+LPS addition, respectively. Results are expressed as % of the incorporation of [^{3}H]-thymidine into control cells (13,915 ± 1,348 cpm/dish). Values are the means ± SD of triplicate experiments

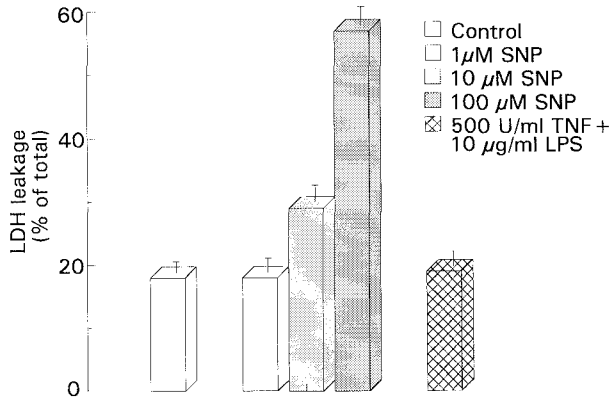

Fig. 3. LDH release in cultured cardiomyocytes treated with TNF+LPS or SNP. Confluent cardiomyocytes were incubated for 20 h with fresh serum-free DMEM in the absence (Control) or presence of 500 U/ml TNF+10 μg/ml LPS or 1, 10 or 100 μM SNP. The LDH leakage into the medium is expressed as percent of total LDH activity, as described in Methods. Values are the mean ± SD of triplicate experiments

staurosporine, an universal trigger of apoptosis (Jacobson et al., 1994), SNP did not produce any evident DNA fragmentation.

Discussion

NO is a ubiquitous molecule involved in different signal transduction pathways leading to either cytotoxicity or cytoprotection depending on the cell type. In fact, NO can induce apoptosis in chondrocytes (Blanco et al., 1995), smooth muscle cells (Nishio et al., 1996) and macrophages (Sarih et al., 1993)

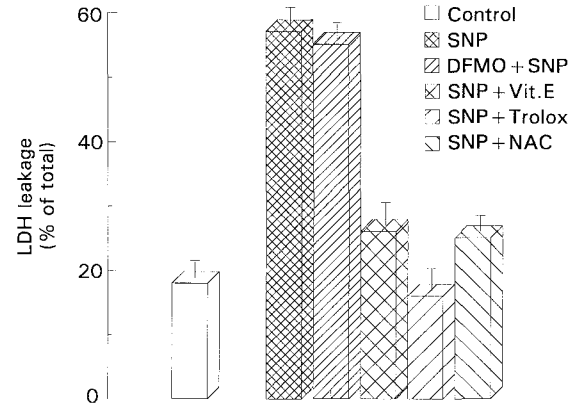

Fig. 4. LDH release in cultured cardiomyocytes treated with 100μM SNP: effect of DFMO or antioxidants. Confluent cardiomyocytes were incubated for 20 h in serum free DMEM in the absence (Control) or presence of 100μM SNP. 4 mM DFMO, 100μM vitamin E (*Vit.E*), 10 mM Trolox or 10 mM N-acetylcysteine (*NAC*) were added together with SNP. The LDH leakage into the medium is expressed as percent of total LDH activity, as described in Methods. Values are the mean ± SD of triplicate experiments

or inhibit the programmed cell death of human eosinophils (Beauvais et al., 1995). In neuronal cells NO may have either protective or cytotoxic properties, depending on the environmental redox potential (Lipton et al., 1993). Our data suggest that, in chick embryo cardiomyocytes, NO formed via NOS induction as well as polyamines synthetized through ODC induction by TNF and LPS are both factors favouring cell growth. On the contrary, NO exogenously supplied as SNP, by acting as negative feedback modulator of inducible NOS and counteracting ODC induction, prevents the onset of DNA synthesis. Moreover, a prolonged exposure of cardiomyocytes to SNP alone causes cell death in a dose-dependent manner. It is noteworthy that the effect of exogenously supplied NO on cardiomyocyte death is not affected by DFMO, while it is inhibited by antioxidants. This indicates that the cytotoxicity of SNP is not dependent on new polyamine biosynthesis, while it could involve oxidative reactions. The formation of highly toxic compound, such as peroxynitrite, which is known to cause oxidative DNA damage (Inoue and Kawanishi, 1995) as well as to induce cell death (Salgo et al., 1995; Lin et al., 1995) might also be taken into account. Interestingly, it has recently been reported that the antioxidant Trolox inhibits thymocyte apoptosis induced by peroxynitrite (Salgo and Pryor, 1996). However, TNF too causes the formation of mitochondrial reactive oxygen intermediates (Goossens et al., 1995), even if this effect has been recently debated (Gardner and White, 1996). Moreover, TNF treatment also stimulates mechanisms of cellular self-protection, such as an increased expression of superoxide dismutase (Hirose et al., 1993). Multiple intracellular pathways are involved in TNF signaling. Among the different effects of TNF there is the activation of protein kinases, such as extracellular signal-regulated kinases (Van Lint et al., 1992; Kyriakis

and Avruch, 1996), leading to a wide range of biochemical activities, depending on cell type and growth state. The cellular receptor for TNF, which is homologous to Fas/Apo-1, can transduce different signals stimulating either apoptosis (Golstein et al., 1991) or proliferation (Hurme, 1988), indicating that, in those cells where TNF is mitogenic, the death signal is converted into a proliferative response. The results of our study indicate that in cardiomyocytes ODC induction by TNF and LPS is not involved in the death signal, as suggested for other experimental systems and that distinct pathways would mediate the response of heart cell to NO. These might lead either to DNA synthesis, when NO is formed through NOS induction, or to cell death, when it is derived, in high concentration, from an exogenous source. Therefore, in cardiomyocytes the ability of NO to either stimulate cell proliferation or induce cell death might depend on new polyamine biosynthesis and on the balance between the availability of NO released and the presence of reactive oxygen species, as well as intracellular antioxidant mechanisms. The balance between NO and superoxide generation has been implicated as a crucial determinant in the aetiology of many human diseases (Darley-Usmar et al., 1995).

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