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The opposing effects of endothelin-1 and C-type natriuretic peptide on apoptosis of neonatal rat cardiac myocytes

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Abstract

C-type natriuretic peptide (CNP) and endothelin-1 are paracrine peptides with opposing effects on cardiac myocyte contraction and intracellular cGMP production. Elevated levels of both endothelin-1 and CNP are found in patients with congestive heart failure. These factors may be related to positive and negative regulation of cell apoptosis in the failing heart. To evaluate the effect of CNP and endothelin-1 on apoptosis of cardiac myocytes and the possible mechanisms involved, primary cardiac myocytes were prepared from neonatal Sabra rats. Cardiomyocyte apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and Annexin V in situ staining. The TUNEL method was used to measure the apoptotic index. CNP and the cGMP derivative, 8-br-cGMP, induced apoptosis of cardiac myocytes. CNP-induced apoptosis could be blocked by HS 142-1 (a mixture of 20–30 kinds of linear β-1, 6-glucan esterified by capronic acid, an antagonist of type A and B natriuretic peptide receptors), and KT 5823 ($C_{29}H_{25}N_3O_5$, the inhibitor of cGMP-dependent protein kinase). α-Difluoromethylornithine (DFMO), the irreversible inhibitor of ornithine decarboxylase, also induced apoptosis to a similar extent. CNP and 8-br-cGMP caused a marked reduction of intracellular ornithine decarboxylase expression, as determined by Western blot analysis and immunohistochemical assay. Preincubation with endothelin-1 attenuated CNP- and 8-br-cGMP-induced cardiomyocyte apoptosis. Endothelin-1 also antagonized the CNP- and 8-br-cGMP-induced reduction of intracellular ornithine decarboxylase expression. These results suggest that CNP has a proapoptotic effect on neonatal rat cardiac myocytes. The effect is mediated via natriuretic peptide receptors and is due to an elevation of intracellular cGMP, which reduces the expression of intracellular ornithine decarboxylase and probably the production of polyamines. Endothelin-1 protects cardiac myocytes against CNP-induced apoptosis by influencing the cGMP-dependent pathway, and this effect is probably mediated through both a reduction of cGMP and antagonism of the CNP-induced reduction of intracellular ornithine decarboxylase expression.

Keywords: Apoptosis; Natriuretic peptide; Endothelin; Cardiac myocyte; Ornithine decarboxylase

1. Introduction

Apoptosis is a gene-directed cell death process (Kerr, 1971) that can be initiated by both exogenous (such as cytokine stimuli) and endogenous (such as DNA damage) signals. Dysregulated apoptosis has been implicated in various cardiovascular diseases (Haunstetter and Izumo, 1998). Among them, loss of cardiac cells by apoptosis had been considered to contribute to the onset and deterioration of heart failure (Olivetti et al., 1997).

Elevated levels of natriuretic peptides are found in patients with cardiac failure. The main effects of these peptides are vasodilatation and natriuresis mediated through their action on a family of three receptor types, of which two are cyclic GMP (cGMP) coupled receptors (Drewett and Garbers, 1994). Activation of these receptors by natriuretic peptides raises intracellular cGMP and subsequently activates a cGMP-dependent protein kinase. We have previously shown that C-type natriuretic peptide (CNP) has a negative inotropic effect on neonatal rat cardiomyocytes, and that this effect is natriuretic peptide receptor specific and mediated through cGMP and protein kinase G (PKG) (Nir et al., 2001). Atrial natriuretic peptide (ANP) has been shown to induce cell cycle entry in terminally differentiated chick cardiac myocytes (Koide et al., 1996). It has also been

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shown that ANP causes apoptosis in a model of neonatal rat cardiomyocytes, an effect that is likewise mediated by cGMP (Wu et al., 1997). However, the role of CNP in cardiac myocyte apoptosis remains unknown.

Endothelin-1 is a peptide derived mainly from endothelial cells and its plasma and myocardial levels are markedly increased in heart failure (Yanagisawa et al., 1988; Wei et al., 1994). It is a potent vasoconstrictor and a potent promitogenic agent (Hirata et al., 1989; Takuwa et al., 1989; Ito et al., 1991). Endothelin-1 is regarded as a survival factor for myocytes as it induces cardiomyocyte hypertrophy (Ito et al., 1991; Shubeita et al., 1990) and protects against beta-adrenergic agonist-induced cardiomyocyte apoptosis (Araki et al., 2000). We have previously shown that endothelin-1 antagonizes the negative inotropic effect of CNP on neonatal rat cardiomyocytes (Fixler et al., 2001).

The objective of the present study is to determine the effect of CNP and endothelin-1 on apoptosis of cardiac myocytes and to evaluate the possible mechanisms involved in the process.

2. Methods

2.1. Cell cultures

Myocardial cells from ventricle fragments of hearts of 1-day-old Sabra rats were isolated by serial trypsinization as described by Hallaq et al. (1989). Cells were suspended in F-10 medium (Beit Haemek, Israel) containing 20% heat-inactivated fetal calf serum (Beit Haemek) and penicillin–streptomycin–Gentamycin antibiotic solution. This medium was also used as the standard culture medium in the experiments. The cell suspensions were enriched with myocytes by preplating on tissue culture plastic Petri dishes for 30 min to allow attachment of fibroblasts to the Petri dish. The cells were plated on 8-well glass slides (permanox, Lab-Tek, Nalge Nunc, Naperville, IL) at a density of 3×10^5 cells/ml and used for apoptosis assay and immunohistochemical assay of ornithine decarboxylase. The cells were plated on Petri dishes (Falcon, polystyrene) at a density of 1×10^6 cells/ml and used for Western blot analysis. Cells were maintained in humidified 5% CO_2 95% air atmosphere at 37 °C. Confluent, spontaneously beating cells were used on the third day of culture for all experiments described herein. The myocardial origin was confirmed by immunohistochemical detection using mouse anti-rat—α sarcomeric actin (Sigma, St. Louis, MO).

2.2. Determination of cardiac myocyte apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

The cardiac myocytes grown on 8-well dishes were incubated for 24 h in the presence of various pharmacological agents. DNA oligonucleosome fragments were assessed using a variation of the method described by Gavrieli et al. (1992). After fixation with 4% paraformaldehyde, DNA breaks were labeled with a dUTP–digoxigenin complex. These dUTP tails were detected using sheep antidigoxigenin antibody labeled with fluorescein. The nuclei were counterstained using propidium iodide 0.5 μg/ml in antifade solution (ApopTag kit, Intergen, Purchase, NY). Analysis was performed on a Zeiss UV-laser confocal microscope with the appropriate filters. For the TUNEL method, an apoptotic index (%) was defined as the number of apoptotic nuclei/number of total nuclei × 100. For each slide of a specific experiment, 8–15 fields were randomly chosen for counting by an investigator unaware of the precise nature of the experiment. The apoptotic index was determined for each slide after counting at least 30 nuclei. For each specific experiment ($n = 1$), at least three slides were prepared.

2.3. Determination of cardiac myocyte apoptosis by in situ staining with Annexin V

To avoid the possible pseudopositive TUNEL reaction caused by necrosis (Ohno et al., 1998), the specificity of the apoptotic effect was further tested using the Annexin V method. Exposed phosphatidyl serine on the cell membrane was detected using a naturally occurring ligand, Annexin V, labeled with fluorescein (Jinshyun et al., 1997) (Annexin V-FITC kit, Bender Medsystem, Vienna). Counterstaining for nuclei was performed using propidium iodide and then followed by fixation using 4% paraformaldehyde. Thus, cells exposing phosphatidyl serine on their outer membrane, but in which the membrane was intact (e.g. apoptotic cells), had membrane Annexin V staining but no nuclear staining. Necrotic cells, in which the cell membrane was ruptured, exhibited both membrane Annexin V staining and nuclear propidium iodide staining. Analysis was performed on a Zeiss UV-laser confocal microscope with the appropriate filters.

2.4. Ornithine decarboxylase assay by Western blot analysis

Ornithine decarboxylase is the rate-limiting enzyme of the polyamine pathway, a pathway closely involved in cell growth, injury and apoptosis. The possible role of ornithine decarboxylase in CNP-induced apoptosis was tested. Three days after cell plating, the culture medium was changed for 24 h to 1% fetal calf serum F-10 medium (to induce "starvation"). Then, cells were incubated at 37 °C in the presence of various pharmacological agents for another 24 h. Cells were homogenized with lysis buffer (Biolabs, Beverly, MA), collected and centrifuged. Then 70 μg of protein extract was subjected to immunoblot analysis (Burnette, 1981) by using rabbit polyclonal antiornithine decarboxylase, for which the specificity was checked during sample preparation (Wang et al., 1999). Peroxidase-conjugated goat anti-rabbit polyclonal antibody (Jackson, West

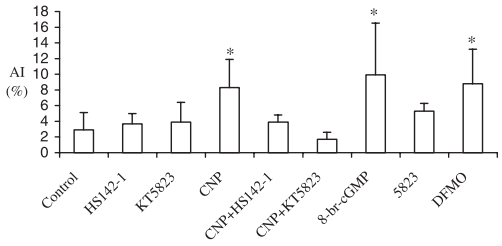

Fig. 1. Apoptotic index (AI%, means ± S.D.) observed by the TUNEL method following pretreatment of cultured neonatal rat cardiac myocytes with various pharmacological agents, as indicated. CNP (10^{-7} M)- and 8-br-cGMP (5×10^{-5} M)-increased cardiac myocyte apoptosis was blocked or attenuated by HS 142-1 (2.5×10^{-5} M) or KT 5823 (10^{-6} M). DFMO (5×10^{-3} M) also induced increased apoptosis. *$P < 0.05$ (ANOVA).

Grove, PA) was used as the primary and secondary antibodies. The specific signal was detected with an enhanced chemiluminescence system (Pierce, Rockford, IL).

2.5. Ornithine decarboxylase assay by immunohistochemical detection

Cardiac myocytes grown on 8-well dishes were washed with 1× phosphate-buffered saline (PBS) once. Paraformaldehyde (4%) was used for fixation of the cells and 0.2% Triton X-100 (in PBS) was used to increase the permeability of cells for 5 min, followed by blocking with 1% bovine serum albumin (Sigma) for 1 h. Following overnight incubation of the cells with rabbit antiornithine decarboxylase antibody (1:400 in 1% bovine serum albumin/PBS) at 4 °C, the cells were exposed to fluorescent iso-thio-cyanate (FITC)-conjugated goat anti-rabbit antibody (Jackson, 1:100) at 37 °C for another 1 h (Wang et al., 1999). The nuclei were counterstained using propidium iodide. Analysis was performed on a Zeiss UV-laser confocal microscope with appropriate filters.

2.6. Research protocols

Seventy-two hours after cell plating, the cells were treated with various agents at 37 °C for 24 h prior to analysis. The agents included standard medium as control, CNP (10^{-7} M, Sigma), endothelin-1 (10^{-7} M, Sigma), CNP plus endothelin-1 (both 10^{-7} M), 8-br-cGMP (a cell-permeable cyclic GMP derivative) (5×10^{-5} M, Sigma), 8-br-cGMP (5×10^{-5} M) plus endothelin-1 (10^{-7} M), HS 142-1 (*) (2.5×10^{-5} M), CNP (10^{-7} M) plus HS 142-1 (2.5×10^{-5} M), KT 5823 (10^{-6} M) ($C_{29}H_{25}N_3O_5$, an inhibitor of cGMP-dependent protein kinase, Calbiochem), CNP (10^{-7} M) plus KT 5823 (10^{-6} M) and DFMO (5×10^{-3} M) (irreversible inhibitor of ornithine decarboxylase, Sigma). Following the preincubation, TUNEL or Annexin V staining, immunohistochemical assay or Western blot analysis for ornithine decarboxylase was performed.

*HS 142-1, a mixture of 20–30 kinds of linear β-1, 6-glucan esterified by capronic acid from culture broth of *Aureobasidium* sp., is an antagonist of types A and B natriuretic peptide receptors and was a gift from Prof. J.C. Burnette Jr. (The Cardiorenal Laboratory, Mayo Clinic, Rochester, MN, USA) (Morishita et al., 1991).

2.7. Statistical analysis

Three to six experiments with three replicates in each experiment were performed. Results are reported as means ± standard deviation (S.D.). Comparison between two groups was done using Student's unpaired *t*-test. Comparison between more than two groups was done using analysis of variance (ANOVA). $P < 0.05$ was considered significant.

3. Results

3.1. Myocyte apoptosis

Incubation of neonatal cardiac myocytes with CNP (10^{-7} M) for 24 h resulted in a significant increase in both TUNEL-positive and Annexin V-positive cardiomyocytes in the CNP-treated group. A summary of TUNEL experiments investigating CNP-induced cardiomyocyte apoptosis, expressed as apoptotic index (AI), is presented in Fig. 1. A significant ($P < 0.001$) increase in AI from 2.9 ± 2.2% (control conditions) to 8.3 ± 3.7% was observed following a 24-h preincubation with 10^{-7} M CNP. Co-preincubation with CNP (10^{-7} M) and HS 142-1 (2.5×10^{-6} M), a specific natriuretic peptide receptor antagonist, yielded an AI of 3.7 ± 1.3%, similar to that observed in the control or HS 142-1 alone conditions. This indicates that CNP-induced cardiomyocyte apoptosis is mediated by a specific receptor.

The fact that CNP induces intracellular cGMP accumulation and that HS 142-1 can abolish this effect (Nir et al., 2001) prompted us to assess the role of 8-br-cGMP, a cell-permeable cGMP analogue. As shown in Fig. 1, 8-br-cGMP increased AI to 9.9 ± 6.6%, which is similar to that of the CNP-treated culture.

We next examined the effect of KT 5823, a cGMP-dependent protein kinase inhibitor, on CNP-induced myo-

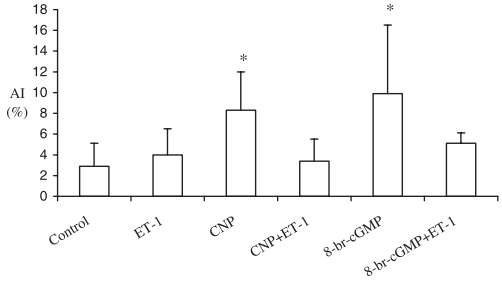

Fig. 2. Apoptotic index (AI%, means ± S.D.) observed by the TUNEL method following coincubation of cultured neonatal rat cardiac myocytes with endothelin-1 (ET-1) (10^{-7} M) and CNP (10^{-7} M) or 8-br-cGMP (5×10^{-5} M). Endothelin-1 blocked or attenuated the increased apoptosis induced by CNP or 8-br-cGMP. *$P < 0.05$ (ANOVA).

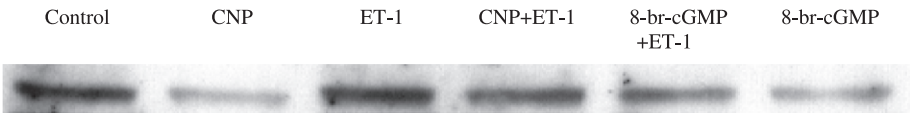

Fig. 3. Western blot analysis of ornithine decarboxylase expression in cardiac myocytes. The cultured cardiomyocytes were exposed for 24 h to various agents as indicated and subjected to immunoblotting. Note reduced expression of ornithine decarboxylase induced by CNP (10^{-7} M) or 8-br-cGMP (5×10^{-5} M). Endothelin-1 (10^{-7} M) blocked this effect of CNP.

cyte apoptosis. As shown in Fig. 1, the apoptotic index after a 24-h incubation with KT 5823 (10^{-6} M) alone, KT 5823 (10^{-6} M) plus CNP (10^{-7} M) and KT 5823 (10^{-6} M) plus 8-br-cGMP (5×10^{-5} M) was $3.9 \pm 2.5\%$, $1.7 \pm 0.9\%$ and $5.3 \pm 1.0\%$ respectively. These experiments indicated that CNP induces neonatal cardiomyocyte apoptosis via a cGMP-dependent protein kinase pathway.

Further to our previous observations on the physiological and biochemical antagonism between CNP and endothelin-1 (Fixler et al., 2001), we conducted experiments looking at cardiomyocyte apoptosis.

A 24-h coincubation with 10^{-7} M endothelin-1 and 10^{-7} M CNP abolished CNP-induced apoptosis, as revealed by TUNEL and Annexin V staining ($AI = 3.4 \pm 2.1\%$ vs. $AI = 8.3 \pm 3.7\%$ respectively, $P < 0.001$). Endothelin-1 also attenuated 8-br-cGMP-induced apoptosis ($AI = 9.9 \pm 6.6\%$ vs. $5.1 \pm 1.0\%$, $P > 0.05$) (Fig. 2).

3.2. Possible role of polyamine pathway in CNP-induced myocyte apoptosis

The possible role of polyamine metabolism in CNP-induced apoptosis was examined in studies of ornithine decarboxylase. A 24-h incubation of cardiac myocytes with 5×10^{-3} M α-difluoromethylornithine (DFMO), the irreversible inhibitor of ornithine decarboxylase, resulted in a significant increase in AI to $8.8 \pm 6.6\%$ ($P < 0.001$), similar to that induced by CNP and 8-br-cGMP (Fig. 1). This experiment suggested that inhibition of ornithine decarboxylase can lead to cardiomyocyte apoptosis, and raises the possibility that CNP induces cardiomyocyte apoptosis by inhibiting the expression or activity of ornithine decarboxylase.

The effect of CNP and 8-br-cGMP on ornithine decarboxylase expression was therefore studied using Western

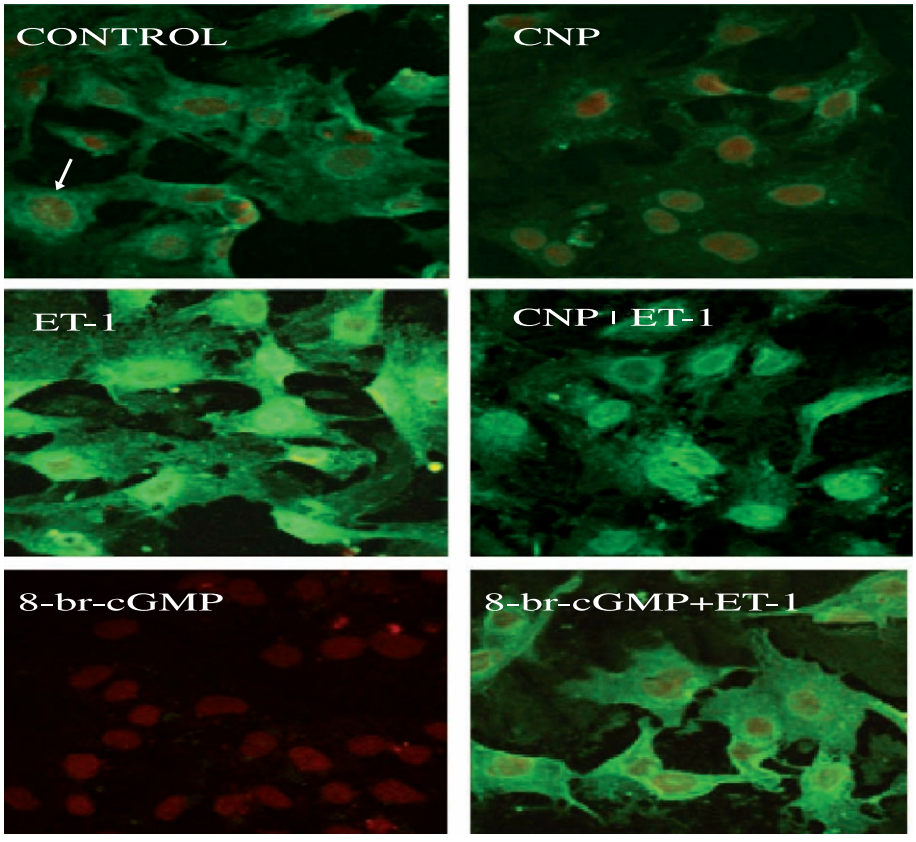

Fig. 4. Immunohistochemistry assay of ornithine decarboxylase expression in cultured rat cardiac myocytes (green discoloration) (arrow). The cultured cardiomyocytes were exposed for 24 h to various agents, as indicated. A reduction of cytosolic staining for ornithine decarboxylase was observed in CNP (10^{-7} M)- and 8-br-cGMP (5×10^{-5} M)-treated cells. This effect was reversed by co-preincubation with endothelin-1 (10^{-7} M).

blot analysis. As shown in Fig. 3, a substantial decrease in the level of ornithine decarboxylase was observed following exposure of cardiomyocytes for 24 h to either CNP (10^{-7} M) or 8-br-cGMP (5×10^{-5} M). When endothelin-1 (10^{-7} M) was added to CNP or 8-br-cGMP, the ornithine decarboxylase level remained similar to that of the control (Fig. 3).

To ensure the validity of these results, intracellular ornithine decarboxylase expression was also evaluated using an immunohistochemical assay. As shown in Fig. 4, cells treated with CNP (10^{-7} M) or 8-br-cGMP (5×10^{-5} M) showed reduced cytosolic staining for intracellular ornithine decarboxylase (Fig. 4B and E). Endothelin-1 (10^{-7} M) abolished the reduced cytosolic staining of ornithine decarboxylase induced by CNP or 8-br-cGMP (Fig. 4D and F).

4. Discussion

The present study reveals that CNP has a proapoptotic effect on neonatal cardiac myocytes. This effect is receptor specific and mediated via a cGMP-dependent protein kinase G-stimulated pathway. This finding is in accord with the general role played by the natriuretic peptide family in cardiac remodeling, since ANP has been reported to induce apoptosis in cardiac myocytes (Wu et al., 1997) and to attenuate hypertrophy induced by catecholamine (Calderone et al., 1998) in neonatal cardiomyocytes.

Endothelin-1 inhibits CNP-induced cardiomyocyte apoptosis. This effect is mediated probably via attenuation of both cGMP production (Fixler et al., 2001) and its effect, since endothelin-1 can also attenuate 8-br-cGMP-induced apoptosis. This effect is not mediated through endothelin-1 receptors since neither endothelin ET_A receptor antagonist (endothelin-16-21) nor ET_B receptor antagonist (endothelin-11-21) inhibited the effect of endothelin-1 on CNP-induced cGMP production (unpublished observation). The interaction of endothelin with natriuretic peptides has been reported in several studies. In a model of vascular smooth muscle cells, angiotensin II- and arginine vasopressin-induced endothelin release could be inhibited by natriuretic peptides (Hanehira et al., 1997). CNP was the most potent inhibitor (CNP>ANP>BNP), and 8-br-cGMP had a similar effect. While endothelin increases ANP and BNP production and/or release in atrial myocytes (Leskinen et al., 1997), it inhibits their cGMP-generating effect. A previous study of neonatal rat cardiomyocytes showed that endothelin-1 inhibited CNP-induced intracellular cGMP accumulation as well as the negative inotropic effect induced by CNP and 8-br-cGMP (Fixler et al., 2001). The present study is the first to demonstrate the interaction of endothelin-1 with CNP on cardiomyocyte apoptosis. Since levels of both endothelin-1 and CNP are elevated in congestive heart failure, the present finding of opposing effects of endothelin-1 and CNP on cardiac myocyte apoptosis and the possible imbalance between them may contribute to our understanding of the pathogenesis of congestive heart failure.

Polyamines, which mainly serve to stabilize DNA chains in normal cell metabolism, are involved in the processes of cell growth, cell injury and apoptosis (Choi et al., 2000). Ornithine decarboxylase is the rate-limiting enzyme in polyamine biosynthesis. The ornithine decarboxylase gene is now recognized as a proto-oncogene required for cell-cycle progression and transformation (Pegg et al., 1995). Ornithine decarboxylase can be induced by growth stimuli (Pegg et al., 1995). Blocking the activity of ornithine decarboxylase with α-difluoromethylornithine (DFMO) can lead to inhibition of growth or to attenuation of myocyte hypertrophy (Bartolome et al., 1980). DFMO was also found to induce apoptosis in a human gastric carcinoma model (Takahashi et al., 2000). The present study demonstrates that inhibition of ornithine decarboxylase with DFMO induces cardiomyocyte apoptosis. Reduced ornithine decarboxylase expression and a possible decrease in polyamine production contribute to the machinery of CNP-induced cardiac myocyte apoptosis. Endothelin-1 protects cardiac myocytes against CNP-induced apoptosis, probably by modifying the production and effect of cGMP level on ornithine decarboxylase expression and thus on the production of polyamines.

Mitogen-activated protein kinase (MAPK)-dependent pathways have been shown to be required for the inhibition of cardiac apoptosis (Sheng et al., 1997). Inhibition of p44/42 MAPK induces cardiomyocyte apoptosis (Araki et al., 2000). Flamigni et al. (1999) proved that crosstalk exists between MAPK and polyamine pathways. Activation of MAPK favors the expression of ornithine decarboxylase, and polyamines, in turn, may induce activation of MAPK. cGMP-elevating agents have been demonstrated to suppress the proliferation of vascular smooth muscle cells by inhibiting the activation of the MAPK pathway induced by epidermal growth factor (Yu et al., 1997). In contrast, endothelin-1 has been shown to stimulate the proliferation of rat vascular smooth muscle cells (Shichiri et al., 2000) and mesangial cells (Herman and Simonson, 1995) by activating the MAPK pathway. Thus, endothelin-1 protects against CNP-induced myocardial apoptosis probably in two ways: by antagonizing CNP-reduced intracellular ornithine decarboxylase expression directly or by influencing MAPK pathways by antagonizing CNP-induced accumulation of cGMP. Thus, the effects of both cGMP and endothelin on MAPK activity may explain the results of the present study.

In summary, our study demonstrates that CNP can trigger apoptosis of neonatal cardiac myocytes via a cGMP-dependent pathway. The polyamine pathway is probably involved in CNP-induced apoptosis. Endothelin-1 can antagonize the CNP-induced apoptotic effect, probably by reducing the production of intracellular cGMP and attenuating its effect on ornithine decarboxylase expression.

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