

## ARTICLES

# Angiotensin Converting Enzyme Inhibition Decreases Cell Turnover in the Neonatal Rat Heart

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### ABSTRACT

The renin angiotensin system plays an important role in growth and development. Exposure of the neonate to an ACE inhibitor increases mortality and results in growth retardation and abnormal development. We have demonstrated that ACE inhibition in the developing kidney increases apoptosis and decreases cell proliferation, which may account for renal growth impairment. To evaluate the role of endogenous angiotensin in cardiac development, the relationship between ACE inhibition, cell proliferation, apoptosis, several modulators of apoptosis (bcl-2, bcl-xl, and clusterin) was examined in the developing rat heart. Thirty-five newborn rat pups were treated with enalapril (30 mg/kg/d) or a vehicle (control group) for 7 d, and hearts were removed for rt-PCR and Western blotting of bcl-2, bcl-xl, and clusterin. An additional 10 rat pups were treated with hydralazine (10 mg/kg/d) or a vehicle, to serve as a hypotensive control. Cell proliferation was determined by PCNA immunostaining, and apoptosis was detected using the total TUNEL technique. Enalapril treatment resulted in a 24% mortality, reduced body weight, and decreased heart weight ( $p < 0.05$ ). Enalapril decreased proliferating myocytes by 23%, and reduced proliferating cardiac interstitial cells by 8.1% ( $p < 0.05$ ). Enalapril also

decreased myocytes apoptosis by 60%, but the proportion of myocytes undergoing apoptosis was 10-fold less than that of proliferating cells. Cardiac bcl-2 mRNA, clusterin mRNA, bcl-2 protein, and bcl-xl protein content were not changed, but clusterin protein expression was decreased by enalapril treatment. Hydralazine did not alter cardiac cell proliferation or apoptosis. We conclude that ACE inhibition decreases cell turnover in the developing rat heart, which may contribute to cardiac growth impairment. The loss of myocytes may lead to greater myocyte hypertrophy and myocardial damage during later life. (*Pediatr Res* 52: 325–332, 2002)

#### Abbreviations

**ACE**, angiotensin converting enzyme  
**ANG II**, angiotensin II  
**GAPDH**, glyceraldehyde, 3-phosphate dehydrogenase  
**rt-PCR**, reverse, transcriptase PCR  
**TUNEL**, Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling  
**PCNA**, proliferating cell nuclear antigen  
**RAS**, renin, angiotensin system

The renin angiotensin system (RAS) plays a role in the maintenance of systemic arterial pressure in the newborn, and provides a homeostatic response to hypovolemia (1). Davidson (1) demonstrated parallel increases in plasma renin activity in arterial ANG-II concentration occurred in the immediate minutes after delivery. Furthermore, the release of catecholamines may be stimulated by the production of ANG II, and the activation

of the sympathoadrenal system at birth has been implicated in several adaptive processes and structural integrity (2).

In newborn animals and human infants, the activity of the RAS increases at birth, which is important in regulating cellular growth and organ differentiation (1, 3). ANG-II, which is a final product of the RAS, has been reported to exert atrophic effect on cardiac myocytes in cultures (4) and *in vivo* (5). ANG-II may stimulate a growth response in cardiac tissue indirectly, by increased blood pressure and total peripheral vascular resistance, which develops in response to ANG-II receptor coupling in the vasculature (5). Alternatively, ANG-II may have a direct effect by coupling to its cardiac membrane receptor, resulting in the inhibition of protein synthesis in

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cardiac cells by unknown mechanisms (5). A direct role for ANG-II in normal cardiac and vascular growth and abnormal cardiovascular growth observed in primary hypertension is supported by the discovery of an intrinsic cardiac RAS within the blood vessel wall. As shown previously, octapeptide ANG-II directly stimulated protein synthesis and cell growth in embryonic chick myocytes in cell culture (4). The actions of ANG-II in the late suckling period may be a critical determinant of long-term cardiovascular functions in animals (6). Treatment with the ACE inhibitor has cardioprotective effects in animal models with regional myocardial ischemia and has been used effectively in the treatment of congestive heart failure, cardiac hypertrophy, coronary atherosclerosis, myocardial infarction, and postinfarction remodeling (7–10).

Growth and development of individual organs are the result of a balance between cellular proliferation and cell death. However, the significance of cell death has been relatively ignored, and only a few studies have directed attention to the cardiovascular system. Apoptosis, or programmed cell death, is essential for normal development, and dysregulation of the process can lead to a spectrum of defects ranging from embryonic lethality to tissue specific perturbation of postnatal development. In addition to its well-documented effects on cellular proliferation and hypertrophy, ANG-II is also now recognized to modulate apoptosis (11). The oncoproteins, *bcl-2* and *bcl-x*, initially discovered in B-cell lymphoma, have been reported to play a role in the prevention of apoptosis. The *bcl-2* proto-oncogene is a regulatory protein molecule related to apoptosis, and has been shown to suppress apoptosis in a number of model systems (12). Clusterin is another apoptosis related molecule whose precise physiologic or pathologic role remains poorly defined. Clusterin appears to play a major role in promoting epithelial cell aggregation and in preventing apoptosis (13). Clusterin has also been shown to play important roles not only in remodeling damaged tissues, but also in tissue reorganization during embryonic development (14, 15).

The cellular mechanism underlying angiotensin dependent stimulation of cardiac development in apoptosis *in vivo* has not yet been elucidated. The present study was therefore designed to investigate the role of endogenous ANG-II in the cellular proliferation and apoptosis of the neonatal rat heart. The experiment and approach consisted of inhibition of endogenous ANG-II production by chronic administration of Enalapril, an ACE inhibitor, to neonatal rat pups. In addition, the consequences of ACE inhibition on known modulators of apoptosis (*bcl-2*, *bcl-x*, and clusterin) were investigated.

## METHODS

**Animal preparation.** Forty five neonatal rat pups from five pregnant Sprague Dawley rats were breastfed by their own mother throughout the study. Body weights were measured daily from birth and a dose of 30 mg/kg of enalapril (Sigma Chemical Co., St. Louis, U.S.A.) (E group) and water (control group) were fed *via* an orogastric tube. A dose of 30 mg/kg of enalapril is known to block the effects on angiotensin I (16). After 7 d their hearts were harvested and RNA analysis, protein assays, detection of apoptosis and immune histochemistry were

performed. The experimental protocol was approved by the Animal Care Committee of Korea University Guro Hospital.

**Effect of hydralazine in newborn rats.** According to the methods of Tufro-McReddie (17), Sprague Dawley pups were given hydralazine ( $n = 5$ ) at a s.c. dose of 10 mg/kg daily from birth to 7 d of age, and compared with control littermates receiving saline ( $n = 5$ ). This dose of hydralazine has been shown to reduce blood pressure significantly (18, 19). At the end of the study period, rats were killed, kidneys were harvested, and PCNA – positive and apoptotic cells were identified as described below.

**Immune histochemical stain.** To assess cell proliferation, an immune histochemical stain for proliferating cell nuclear antigen (PCNA) was carried out. Harvested hearts were treated in 10% formalin or Bouin solution (Sigma Chemical Co., St. Louis, U.S.A.) and embedded in paraffin. PCNA-positive cells were detected using the avidin-biotin immunoperoxidase method (Vectastain ABC kit, Burlingame, CA, U.S.A.). Primary antibodies were monoclonal anti-mouse PCNA antibody (1:100 dilution, DAKO, Glostrup, Denmark) in the study group and PBS (PBS) in the negative control group. Each slide was cross-stained with 0.5% methyl green solution (Trevigen, Gaithersburg, MD, U.S.A.). After dehydration, we compared the slides using a light microscope. PCNA positive cells were measured by counting 20 areas ( $25 \times 25 \mu\text{m}$ ) and the average was statistically obtained. In addition to this, microscopic identification ( $400\times$ ) of myocytes and interstitial cells expressing PCNA was performed by two pathologists using a double-blind method. Cells were counted as myocytes if they showed central ovoid nuclei with clear zone and eosinophilic abundant cytoplasm. Cells with spindle-shaped nuclei and elongated modest cytoplasm were counted as interstitial cells. Count was done randomly throughout all the observed fields.

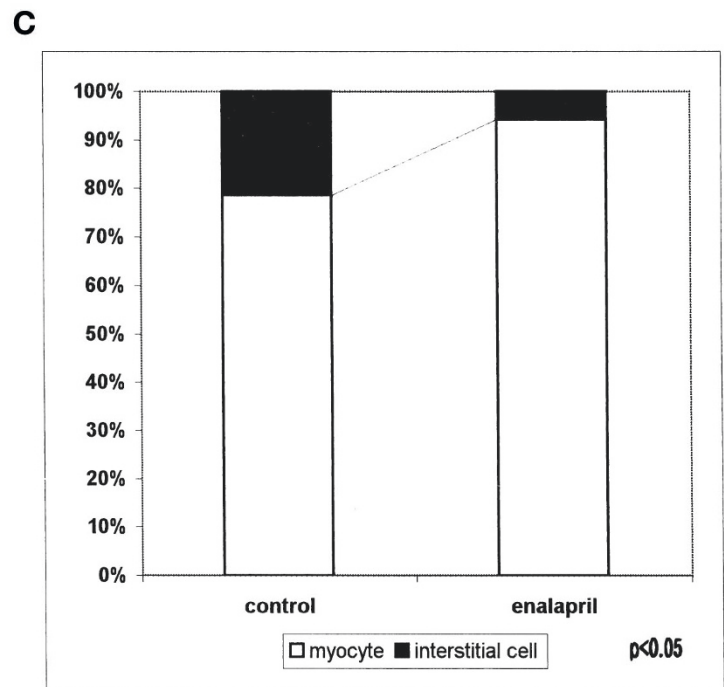
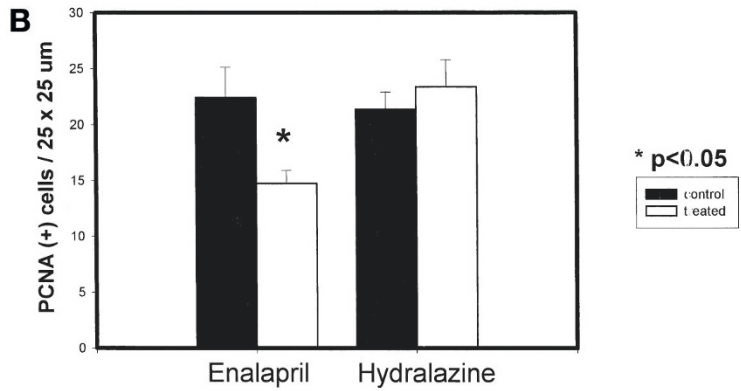
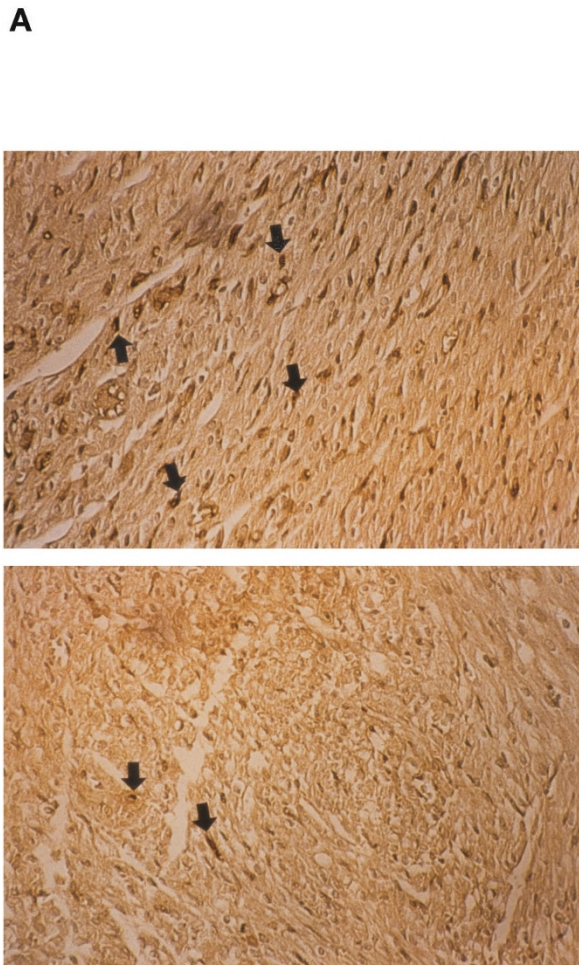
**Detection of apoptosis.** Apoptotic nuclei were labeled using the TACS TM 2 TdT *In situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, U. S. A.). Tissues were fixed in 4% neutral buffered formalin for 4 h at  $4^\circ\text{C}$ , dehydrated in graded alcohols, and embedded in paraffin. The samples were then cut into  $7 \mu\text{m}$  sections and dried onto silanized slides (Sigma Chemical Co., St. Louis, MO, U.S.A.). Slides were deparaffinized and digested for 10 min with proteinase K (20  $\mu\text{g}/\text{ml}$ ) and an endogenous peroxidase activity was quenched in 2% hydrogen peroxide for 5 min. Samples were equilibrated using a provided buffer for 2 min and labeled with biotin dNTP mixtures, TdT,  $\text{CoCl}_2$ , and a labeling buffer according to the manufacturer's advice. Samples were placed inside a humidity chamber in a  $37^\circ\text{C}$  incubator for 60 min. Negative controls were treated with water instead of TdT. The labeling reaction was stopped using the provided stop buffer for 5 min. Samples were rinsed in PBS for 2 min and treated with the provided Streptavidin-Horseradish Peroxidase Detection solution for 10 min and finally washed 2 times in PBS for 2 min each wash. The color was developed using 0.05% DAB and 0.05% hydrogen peroxidase in PBS for 5 min. The sections were rinsed in water and counter-stained in methyl green for 5 min, and the samples were dehydrated and mounted. After dehydration, we compared them using a light microscope. Positive apoptotic cells were measured by counting 20 areas ( $25 \times 25 \mu\text{m}$ ) and

the average was statistically obtained. In addition to this, microscopic identification (400×) of myocytes and interstitial cells stained positively in the TUNEL assay was performed as the same method of PCNA staining. Only morphologically distinct nuclei stained positively by the TUNEL assay were counted as apoptotic. Scattered or clustered apoptotic bodies were not counted as apoptotic due to the lack of identification of cell type.

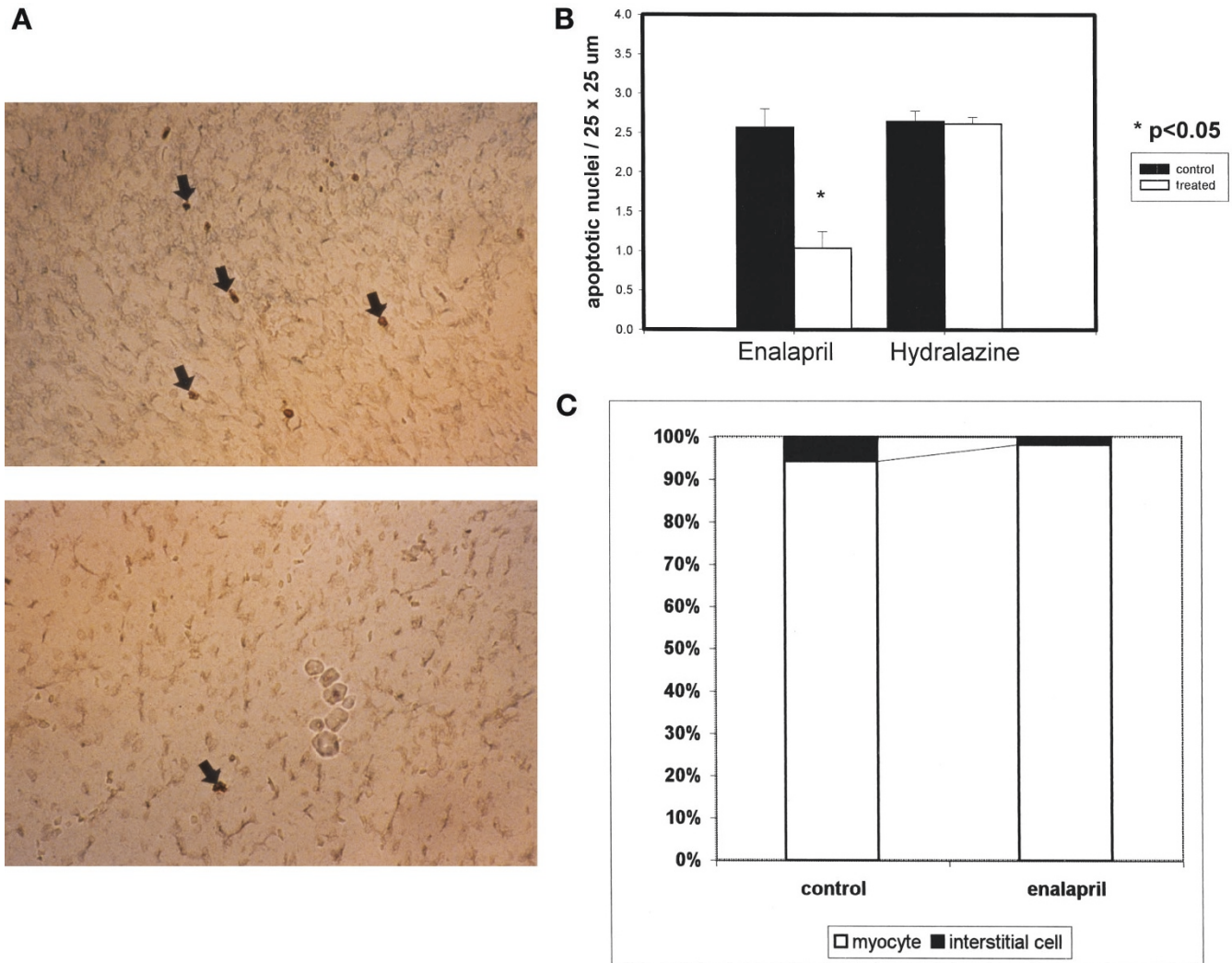
**Isolation of RNA and Analysis of mRNA.** After being removed, the cardiac tissue was frozen in liquid nitrogen and stored at -70°C. Total cellular RNA was isolated using TRI-REAGENT (Molecular Research Center, Cincinnati, USA) and homogenized with tissue tearor (Model 985-370, Biospec products, Bartlesville, OK, U.S.A.). 37% chloroform (200 μL/1 mL TRI reagent) was added to the homogenates and centrifuged at 12,000 rpm for 15 min in 4°C separating into three layers: RNA, DNA, and protein material. The colorless clear upper layer was moved into another Eppendorf tube and

isopropanol was added. This was left at room temperature for 15 min and was then centrifuged at 12,000 rpm for 10 min in 4°C until the white-colored cellular RNA pellet was isolated. It was dried at room temperature for 5 min after washing in 75% ethanol and was dissolved in 25 μL Forma zol (Molecular Research Center, Cincinnati, U.S.A.) at 55°C heating block for 10 min and then stored at -70°C. The RNA was quantified spectrophotometrically by absorbance at 260 nm.

**cDNA synthesis by reverse transcription (RT) and PCR (PCR).** Oligo dT primed 1st strand cDNA was synthesized from the template RNA 1 μg through a cDNA Synthesis Kit (Boehringer Mannheim Corp., Indianapolis, IN, U.S.A.). AMV reverse transcriptase was used for synthesis of first strand cDNA for use in subsequent amplification reactions. In the following PCR reaction with the forward primer 5'-AATGCATCCTGCACCACCAA-3', the reverse primer 5'-GTAGCCATATTCATTGTCATA-3' for GAPDH designed from DNA template of rats, 5'-CGGGTACCGACAAT-



**Figure 1.** PCNA positive proliferating cells in the heart. (A) Immune-histochemical stain for PCNA in both groups shows that proliferating cells in the E group is decreased more than the control group. Black arrows: PCNA positive proliferating cell (× 400, top: control, bottom: E group). (B) Number of PCNA positive cells measured by counting 20 areas, 25 × 25 μm,  $p < 0.05$  (black bar: control, white bar: enalapril or hydralazine treated group). (C) Percentage of PCNA positive myocytes and interstitial cells between control and enalapril treated group,  $p < 0.05$ .



**Figure 2.** Distribution of apoptosis in the myocardium of both group. (A) The distribution of apoptotic cells in the myocardium of the E group is decreased more than the control group, black arrows: apoptotic cells ( $\times 400$ , top: control, bottom: E group). (B) Number of the apoptotic cells measured by counting 20 areas,  $25 \times 25 \mu\text{m}$ ,  $p < 0.05$  (black bar: control, white bar: enalapril or hydralazine treated group). (C) Percentage of apoptotic myocytes and interstitial cells between control and enalapril treated group.

GAGCTCCA-3', 5'-GGCCGCGGCCACTTCTGCAGAC-3' for clusterin, and 5'-AGACAGCCAGGAGAAATCAA-3', 5'-TGGGATACTGGAGATGAAGA-3' for bcl-2, all three 515, 310, and 386 base pairs of the PCR product for each gene were obtained. The PCR was carried out in a different mode of time and temperature for each reaction period using Perkin Elmer Cetus DNA Thermal Cycler (Model 2400, Foster City, CA, U.S.A.).

For GAPDH as a house-keeping gene, PCR was performed at 30 cycles heating for 45 s at  $94^{\circ}\text{C}$  after denaturation for 5 min at  $94^{\circ}\text{C}$ , followed by annealing for 45 s at  $60^{\circ}\text{C}$ , and last a primer extension for 45 s at  $70^{\circ}\text{C}$ . For bcl-2, PCR was performed at 32 cycles heating for 45 s at  $94^{\circ}\text{C}$  after denaturation for 5 min at  $94^{\circ}\text{C}$ , followed by annealing for 45 s at  $62^{\circ}\text{C}$  and last a primer extension for 1 min at  $72^{\circ}\text{C}$ . For clusterin, this was performed at 30 cycles heating for 30 s at  $94^{\circ}\text{C}$  after denaturation for 5 min at  $94^{\circ}\text{C}$ , followed by annealing for 30 s at  $62^{\circ}\text{C}$  and primer extension for 30 s at  $72^{\circ}\text{C}$ . These amplified PCR products were visible as a fluorescent band under UV rays

after agarose gel electrophoresis with a difference of time and ethidium bromide staining. Polaroid photographs were scanned using Epson GT-9500 (Seiko Corp, Nagano, Japan) and quantitated by densitometer (Image PC alpha 9, N.I.H., Bethesda, U.S.A.) and the values were revised by GAPDH.

**Protein extraction.** Alcohol (100%) was added to interface and organic phase remained from separation of RNA, and then the DNA portion was precipitated after being centrifuged at 5000 rpm for 5 min. Isopropanol was added to the remaining ethanol upper layer, which was centrifuged and dissolved in 0.3 M guanidine hydrochloride in 95% ethanol. Afterward, this layer was washed three times and turned into a protein pellet. The extracted protein was dissolved in 1% SDS (SDS) solution and preserved at  $-20^{\circ}\text{C}$ . The Bradford method was used in the quantification of protein.

**Immunoblot analysis.** The extracted proteins were solubilized in  $5 \times$  SDS loading buffer for five minutes at  $95^{\circ}\text{C}$  and separated by electrophoresis on 10% SDS-polyacrylamide gels under reducing conditions. Subsequently the proteins were

transferred to nitrocellulose membranes (Amersham Life Science, Buckinghamshire, England). The nitrocellulose membranes were blocked in 5% skim milk with TBS-T (0.05% Tween 20 in 50 mM Tris, 150 mM NaCl, 0.05% NaN<sub>3</sub>, pH 7.4) at room temperature for 1 h. The membranes were washed two times in TBS-T and incubated for 18 h at 4°C with rabbit polyclonal antiserum (bcl-2: Calbiochem, Cambridge, MA, U.S.A., bcl-xl: Oncogene, Cambridge, MA, U.S.A., and clusterin: Upstate Biotechnology Incorporated, Lake Placid, NY, U.S.A.), each diluted 1:20, 1:400 and 1:100 in TBS-T. Thereafter the membranes were washed two times with TBS-T and incubated for 40 min with an anti-rabbit IgG (Amersham Life Science, Buckinghamshire, England) at room temperature. After washing by TBS-T four times, the secondary antibody bound to the nitrocellulose was detected by incubation for 1 min with a detection reagent (Amersham Life Science, Buckinghamshire, England) and then exposed to medical x-ray film (Agfa, Mortsel, Belgium) for 1 min. The film was developed by an using FPM-3500 Fuji x-ray Film Processor (Fuji, Otawara, Japan). After that, for stripping and reprobing, same membranes were submerged in stripping buffer (2% SDS in 62.5mM Tris-HCL, 0.1 M β-mercaptoethanol) and incubated at 50°C for 10 min with occasional shaking. To control equal loading, α-tubulin (1:1000 dilution, Oncogene) and anti-mouse IgG conjugated horseradish peroxidase (1:1000 dilution, Amersham Life Science, Buckinghamshire, England) were used as primary and secondary antibodies in a same methods as above. The developed X-rays were scanned using Epson GT-9500 (Seiko Corp, Nagano, Japan) and quantitated by a densitometer (Image PC alpha 9, N.I.H., Bethesda, U.S.A.).

**Statistical analysis.** Data are presented as mean ± SEM. Comparisons between the two groups were performed by the Student-Newman-Keuls methods. Statistical significance was defined as  $p < 0.05$ .

## RESULTS

**Mortality rate.** Six among the 25 rats of the Enalapril group (E group) (24.7%) died. In the control group, one of the 20 rats died (5%). The mortality rate was higher in the E group than the control group ( $p < 0.05$ ).

**Body weight and heart weight.** On harvest, the average body weights were  $14.6 \pm 0.5$  g in the E group and  $17.1 \pm 0.6$  g in the control group. Weights of the hearts were  $0.09 \pm 0.01$  g in the E group and  $0.11 \pm 0.00$  g in the control group. Weights of the body and heart were reduced in the E group more than in the control group ( $p < 0.05$ ).

**Proliferating cell nuclear antigen (PCNA).** In the E group, PCNA positive cells were rarely found in the myocardium of the neonatal rat heart as compared with the control group. PCNA-positive cells were  $22.46 \pm 2.69$  of the control group, and  $14.75 \pm 1.19$  of the E group in each field of  $25 \times 25 \mu\text{m}$  ( $p < 0.05$ , Fig. 1A,B).  $78.6 \pm 1.4\%$  of PCNA-positive cells were myocytes, and  $21.4 \pm 1.8\%$  were interstitial cells in the control group, whereas  $94.2 \pm 4.1\%$  were myocytes and  $5.8 \pm 4.1\%$  were interstitial cells in the E group ( $p < 0.05$ , Fig. 1C). The reduction in proliferating myocytes in the E group was

23%, while the reduction in proliferating interstitial cells was 81%.

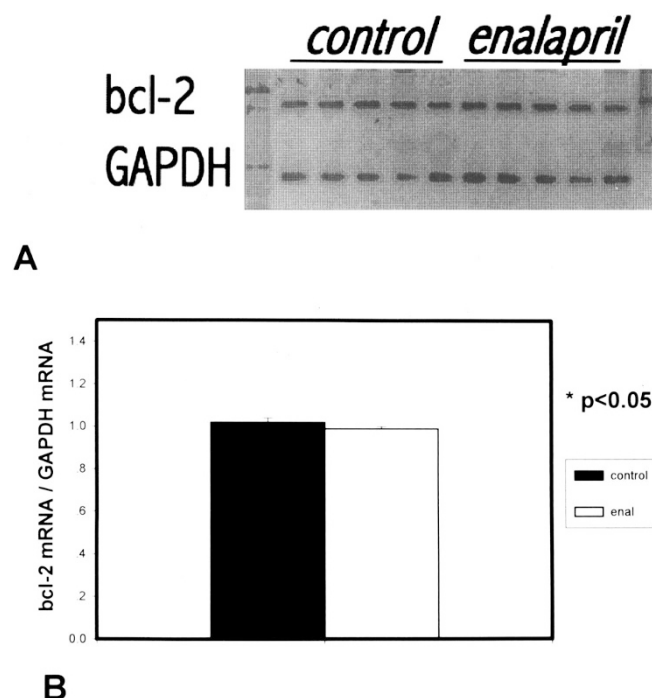
**Apoptosis.** In the E group, apoptotic nuclei were rarely found in the myocardium as compared with the control group. Apoptotic nuclei were  $2.56 \pm 0.23$  of the control group and  $1.04 \pm 0.21$  of the E group in each field of  $25 \times 25 \mu\text{m}$ , a reduction of 60% ( $p < 0.05$ , Fig. 2A,B). In cell type identification,  $94.3 \pm 1.9\%$  of apoptotic cells were myocytes in the control group and  $98.2 \pm 1.2\%$  in the E group ( $p > 0.05$ , Fig. 2C).

**Expression of bcl-2 mRNA and protein.** As a result of semi-quantitative RT-PCR, bcl-2/GAPDH mRNA expressions were  $1.02 \pm 0.02$  in the control group, and  $0.99 \pm 0.01$  in the E group (Fig. 3A,B). In addition, as a result of the immunoblot analysis, bcl-2 protein density was  $62.60 \pm 3.44$  units in the control group, and  $68.0 \pm 5.38$  units in the E group (Fig. 4).

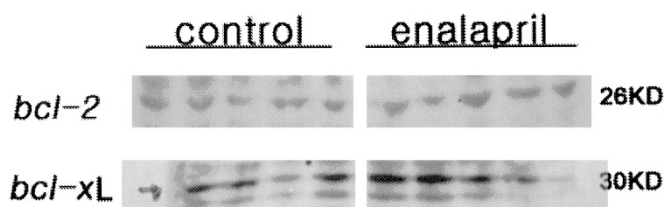
**Expression of bcl-xl protein.** As a result of immunoblot analysis, there was no significant difference between the two groups. Bcl-xl protein density was  $173.25 \pm 23.94$  units in the control group, and  $167.4 \pm 29.59$  units in the E group (Fig. 4).

**Expression of clusterin mRNA and protein.** As a result of semi-quantitative RT-PCR, clusterin/GAPDH mRNA expressions were  $0.90 \pm 0.01$  in the control group, and  $0.92 \pm 0.02$  in the E group (Fig. 5A,B). As a result of the immunoblot analysis, however, clusterin/tubulin protein expressions were decreased significantly in the E group ( $0.41 \pm 0.08$ ) as compared with the control group ( $0.67 \pm 0.05$ ) (Fig. 6A,B,  $p < 0.05$ ).

**Effect of hydralazine in newborn rats.** To determine whether systemic hypotension *per se* alters cardiac cell proliferating

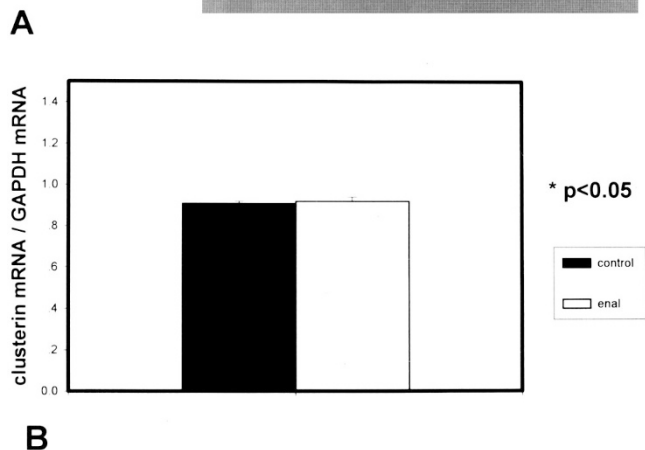
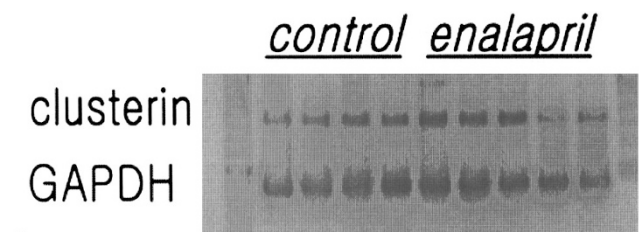


**Figure 3.** Expression of bcl-2 mRNA from the developing heart. (A) Semiquantitative rt-PCR shows that expression of bcl-2 mRNA is not different between the two groups. (B) Expression of bcl-2 mRNA between the two groups is not different (black bar: control, white bar: enalapril treated group).



**Bcl-2 and bcl-x protein expression**

**Figure 4.** Expression of the bcl-2 and bcl-xl proteins from the developing heart in both group. Western blot assay of the bcl-2 and bcl-xl proteins show that there is no difference between the two groups (Top: bcl-2, bottom:bcl-xl).

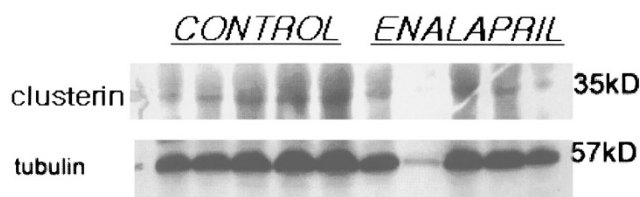


**Figure 5.** Expression of clusterin mRNA from the developing heart (A) Semiquantitative rt-PCR shows that expression of clusterin mRNA is not different between the two groups. (B) Expression of clusterin mRNA between the two groups is not different (black bar: control, white bar: enalapril treated group).

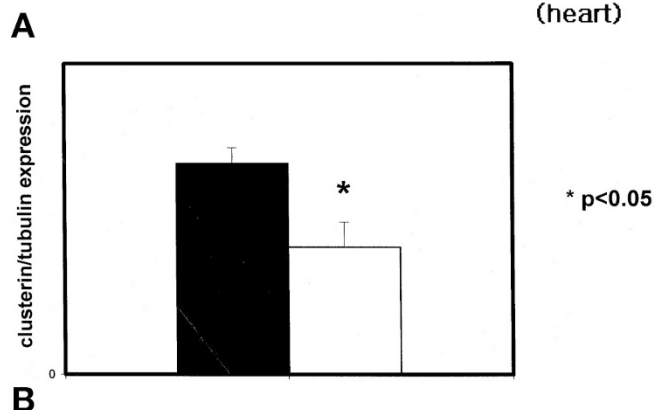
eration and apoptosis, newborn rats were given hydralazine. Hydralazine did not alter cardiac cell proliferation and apoptosis. Hydralazine treated hearts had  $23.4 \pm 2.4$  PCNA positive cells and  $1.65 \pm 0.13$  apoptotic cells, and control hearts had  $21.4 \pm 1.5$  PCNA positive cells and  $1.62 \pm 0.08$  apoptotic cells (Fig. 1B, Fig. 2B).

**DISCUSSION**

A major finding in the present study is the demonstration that chronic ACE inhibition impairs normal postnatal cardiac growth. Development and growth of the heart is controlled by a variety of intrinsic and extrinsic factors. The developing blood volume and pressure in the neonatal heart dictate the hypertrophic stage of normal growth and this may be further modified by abnormal hemodynamic and humoral factors within the myocardium (20). The myocardium is composed of cardiac myocytes and interstitial cells (vascular endothelial



**Clusterin protein expression by Western blotting (heart)**



**Figure 6.** Clusterin protein expression of the developing heart. (A) Western blot assay of the clusterin protein shows that expression of clusterin in the E group is significantly more decreased than the control group (tubulin - a loading control). (B) clusterin/tubulin protein expressions measured by densitometer, black bar: control, white bar: enalapril treated group,  $p < 0.05$ .

cells, vascular smooth muscle cells, fibroblasts, and macrophages). Cardiac myocytes, which form the major functional and structural portion of the heart, can divide only in the fetal and neonatal periods, after which these cells undergo hypertrophy. Cardiac pressure load, in particular during cardiac growth and development, has been identified as an important mechanism regulating the growth of the heart *in vivo* (21). The RAS is involved in the regulation of cardiac growth through this load dependent pathway. ANG-II, as the main effector peptide in the RAS, elicits potent vasoconstriction, causes inotropic and chronotropic changes in the heart, and increases growth of cardiac and vascular smooth muscle cells mainly *via* AT1 receptors (4, 22–24). In addition, it has a stimulatory effect on protein synthesis and on cellular hypertrophy of the heart inducing growth-promoting genes in cardiac myocytes.

Studies in newborn animals and human infants have shown that the activity of the RAS increases at birth compared with that in the adult (1, 3). This increased activity has previously been shown to be important in regulating several of the cardiovascular and autonomic adaptations which occur during the transition from fetal to newborn life (1, 25). More evidence that the RAS may be involved in the regulation of cardiac growth comes from pharmacological studies using inhibitors of the system by blocking the effects of ACE and the ANG-II AT1 receptor. Beinlich *et al.*, (26, 27) found that ANG-II is required for the normal neonatal cardiac development of pigs.

During early development, rat cardiac muscle cells actively proliferate. Shortly after birth, division of cardiac muscle cells ceases, whereas DNA synthesis continues for approximately two weeks at a progressively diminishing rate (28). During the

postnatal period, there is an uncoupling of DNA replication from cell division and rapid polyploidization occurs during the first postnatal week (29, 30). In this study, ACE inhibition diminished the number of proliferating myocytes by 23%, and of interstitial cells by 81%. The reduction in myocyte proliferation by ACE inhibition presumably contributed to the reduction in cardiac mass. It has been shown that blocking ANG-II by either ACE inhibition or AT1 receptor inhibition results in a significant reduction of macrophage and interstitial cell infiltration and proliferation in the adult rat kidney (31, 32). However, there are no reports regarding the relationship between ANG-II and interstitial cells in the developing heart. The precise action of ANG-II on interstitial cell proliferation and regulation on cardiac development needs to be further clarified.

In this study, cardiac apoptosis occurred primarily in myocytes and was decreased 60% by ACE inhibition, but the prevalence of apoptotic cells was approximately 10-fold less than that of proliferating cells. This presumably accounts for the loss in cardiac mass in Enalapril treated rats despite a reduction in apoptosis. Apoptosis is a ubiquitous mode of physiologic cell death, and is also a major factor in the control of organ remodeling during normal and abnormal growth (33, 34). Apoptosis in the heart is only now being recognized (35, 36). The heart undergoes extensive remodeling during embryogenesis, wherein apoptosis significantly contributes to the development of the cardiac chambers and correct routing of the great vessels. Apoptotic myocyte cell death occurs in the early stages of postnatal development in the rat heart, becoming undetectable by 21 d after birth (12). Recently, ANG-II was shown to induce apoptosis in myocytes *via* the activation of surface AT-1 receptors, resulting in an increase in cytosolic calcium and the stimulation of calcium dependent dNASE-1 (37, 38). Angiotensin II stimulated a 2.5-fold increase in apoptosis in neonatal ventricular myocytes (38).

In the present study, both apoptosis and cellular proliferation in the developing rat heart were decreased in the Enalapril treated group. Although blood pressure was not monitored directly in this study, hydralazine treatment was used to control for the effects of hypotension in the observed cellular responses in the developing rat heart. The results that unlike Enalapril, hydralazine did not alter cell proliferation or apoptosis. Our data are in agreement with previous reports in ten to eleven week old rats treated with Enalapril, hydralazine, or calcium channel blockers, showing that the effect of ACE inhibition on growth is not due to changes in blood pressure (39). Hence, these findings suggest that the cellular actions of ANG-II in the developing heart are independent of hemodynamic changes. It may be speculated that this apoptotic activity is mediated by several apoptosis related growth factors and cytokines (40). Bcl-2 proto-oncogene product, which is a protein mainly present in mitochondrial membranes and nuclear envelopes, has been shown to be inversely related to apoptotic cell death in several model systems (12). Apoptosis repressing ability of bcl-2 and bcl-x protein have been related to the neutralizing activity of pro apoptotic effect through the BAX protein which promotes apoptosis as another bcl-2 family member (41, 42). Kajstura *et al.* (12) had found that cardiac myocyte apoptotic

activity in early development increased at 1, 5, and 11 d postnatally, and was no longer detectable at 21 d. They also noted that bcl-2 mRNA levels were high in fetal myocytes, decreased markedly at 1 to 5 d, and progressively increased at 11–21 d. The chronic administration of losartan (an AT1 receptor blocker) is associated with normalization of cardiac apoptosis, BAX protein, and the bcl-2/BAX ratio in treated spontaneously hypertensive rats (but with no significant change in the expression of bcl-2) (43). In our study, no significant changes in the expression of bcl-2 or bcl-x 1 were observed in the developing heart after Enalapril administration. However, apoptotic members of the bcl-2 family can interact, and the overall effect on cell survival depends on the balance between the activity of apoptotic and anti-apoptotic proteins (44).

Clusterin is a glycoprotein produced in response to tissue injury, and it also protects cell membranes and promotes cell aggregation (45). Clusterin is induced in some models of apoptosis and is one of the proteins likely to be involved in the maintenance of cell integrity. While there is no expression of clusterin in cells undergoing apoptosis, clusterin is expressed by surrounding surviving cells during *in vitro* programmed cell death (46). In the present study, although there was no significant effect of Enalapril on cardiac expression of clusterin mRNA, Enalapril decreased clusterin protein in the neonatal rat heart. While not consistent with an anti-apoptotic role for clusterin in this setting, these results suggest that ANG-II modulates clusterin expression in the heart as has been demonstrated in the kidney (47).

In the kidney, the effects of ANG-II on clusterin expression are dependent on the predominant ANG-II receptor type (47). At least two ANG-II receptors subtypes, the AT1 and AT2 receptors, have been identified. In developing rats, both *in situ* hybridization and autoradiographic studies have shown that the AT2 receptor expression is high in the developing embryo, is localized to fetal mesenchymal tissues, and decreases late in development and with postnatal maturation (26, 48–52). The predominance of AT2 receptors during fetal life as led investigators to postulate that these receptors may be involved in protein synthesis and growth (48, 53). ANG-II mediated cell proliferation and maturation of tissue *via* the AT2 receptor is important in the developing organ. Further studies are needed to clarify the downstream signaling mechanisms responsible for ANG-II modulation of apoptosis in the developing rat heart.

## CONCLUSION

In summary, inhibition of endogenous ANG-II in the neonatal rat decreased myocyte proliferation, which may account at least in part for impaired cardiac growth. In addition, ACE inhibition had an even more profound effect to reduce cardiac interstitial cell proliferation. These effects of ACE inhibition were counterbalanced by a reduction in myocyte apoptosis, which, however, accounted for only 10% of the relative fraction of cells undergoing proliferation. The overall effect of ACE inhibition on myocyte turnover was therefore a net reduction in cellular proliferation. Although ACE inhibition reduced the abundance of clusterin in the neonatal rat heart,

steady state gene expression was not affected. Additional studies will be required to elucidate the cellular mechanisms responsible for disruption of cellular proliferation and apoptosis by ACE inhibition in the developing heart. We speculate that a reduction in the number of myocytes may lead to maladaptive hypertrophy of remaining myocytes, and may contribute to cardiac damage later in life.

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