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A genetic variant of ACE increases cell survival: a new paradigm for biology and disease

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Abstract

The human angiotensin converting enzyme (ACE) polymorphism is caused by an Alu element insertion resulting in three genotypes (Alu+/+, Alu+/−, Alu−/−, or ACE-II, ACE-ID, and ACE-DD, respectively), with ACE-II displaying lower ACE activity. The polymorphism is associated with athletic performance, aging, and disease. Population studies, however, were confounding because variants of the polymorphism appeared to fortuitously correlate with health and various pathological states. To clarify the functional role of the polymorphism, we studied its direct effect on cell survival. ACE-II (Alu+/+) human endothelial cells (EC) had lower angiotensin-II levels and 20-fold increased viability after slow starvation as compared to ACE-DD cells (Alu−/−). By RT-PCR, only ACE-II cells expressed the pluripotent/stem cell-maintenance factors nanog, numb, and klotho. ACE inhibition by captopril in ACE-DD cells mimicked the ACE-II genotype. These results provide the first evidence of a functional role for a naturally occurring polymorphism, having broad implications for human biology, longevity, and disease.

Keywords: Alu; Stress; Endothelial cells; Apoptosis; Cytoprotection; I/D polymorphism; Nanog; Klotho; Numb; Starvation

The human angiotensin converting enzyme (ACE) polymorphism is caused by an Alu element insertion in intron 16 of the ACE gene. The Alu insert generated three genotypes (Alu+/+, Alu+/−, Alu−/−, commonly described as II, ID, and DD, respectively), with II individuals displaying lower ACE activity in the bloodstream and in tissues [1]. Countless genetic association studies have implicated this polymorphism with health and disease. However, in many instances, the results were confounding. For example, the II genotype is prevalent in extreme athletes [2], but found in lower frequency in the elderly [3]. On the contrary, the DD genotype predisposes to heart and kidney disease [4,5] but is more frequent in the aging populations [3]. To clarify these biologically confounding results, we studied the direct effect of the ACE polymorphism on human cells in vitro. By exploring its role on cellular mechanisms we hope to provide groundwork for explaining the epidemiological data.

As already stated, the II genotype is associated with lower ACE activity in the human population. We have previously shown that this genotype is protective for atrophic age-related macular degeneration (AMD), a disease involving the cell death of the retinal pigment epithelium [6]. In a subsequent study, we demonstrated that lowering ACE activity with captopril rescued vascular endothelial cells from apoptotic cell death in a novel slow starvation assay [7]. Therefore, we hypothesized that because the ACE-II genotype displays lower ACE activity, ACE-II cells would also be protected. Although the action of pharmacological ACE inhibitors on cell survival was informative, deciphering the direct effect of the ACE polymorphism is more intriguing.

Materials and methods

Cell culture. Primary cultures of human endothelial cells from a variety of tissue sources were purchased from the American Type Culture Collection (ATCC) and grown according to their instructions on tissue culture flasks (USA Scientific, Ocala, FL) coated with 0.2% pig gelatin (Sigma Chemical, St. Louis, MO). Cells were tested from the following tissues of origin: umbilical vein; pulmonary vein; iliac

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vein; abdominal aorta; femoral artery; iliac artery; and pulmonary artery. Growth medium consisted of 10% fetal calf serum (Omega Scientific, Tarzana, CA) in F12K medium (Mediatech, Herndon, VA) supplemented by antibiotics/antimycotics (Invitrogen Life Technologies, Carlsbad, CA) and endothelial cell growth supplement (Sigma).

Immunofluorescent analysis. Endothelial cells were cultured on LabTekII chamber slides (USA Scientific) to approximately 50% confluence, followed by a 3-day treatment with vehicle (phosphate-buffered saline or PBS from Invitrogen) or 1 mM captopril (Biomol, Plymouth Meeting, PA). Cells were fixed and processed for dual immunofluorescence as previously described [7]. Negative controls consisting of secondary antibodies only were used to monitor non-specific staining. High resolution digital images were obtained with a Photometrics Cool SNAP fx digital camera and analyzed using the MetaMorph 6.0r3 software (Universal Imaging, Downington, PA).

Captopril treatment and cell starvation. Confluent EC cultures were treated with vehicle or 1 mM captopril for 2 weeks. Medium was changed two-to-three times per week. "Slow starvation" consisted of not changing the medium until the cells began to die (usually 10–14 days for the cells used in this study) when compared to non-starved controls. Apoptosis was confirmed by DNA laddering whereas successful starvation was assessed by monitoring the appearance of the starvation marker p202 as previously described [7].

Assessment of cell viability. At the end of the starvation period, living cell numbers were calculated from trypsinized cell monolayers combined with the culture medium containing dead cells by trypan blue exclusion (Sigma) as recommended by the manufacturer.

Genomic DNA extraction, polymerase chain reaction, and DNA sequencing. Genomic DNA was isolated from endothelial cells using the Puregene (Minneapolis, MN) DNA isolation kit according to manufacturer's protocol. Total genomic DNA from ACE-II and ACE-DD cells was amplified by polymerase chain reaction (PCR) as previously described [6]. Gel-purified PCR products were sequenced directly by the ABI dye-di-deoxy chain termination cycle sequencing methodology (Laragen, Los Angeles, CA) to confirm the identity of the amplified DNA bands as the Alu insert.

Reverse transcriptase polymerase chain reaction. RNA was isolated from vehicle- or captopril-treated ACE-II and ACE-DD cell cultures at the conclusion of the experiment as previously described [7]. After assessing the quality and quantity of RNA with the Agilent 2100 Bioanalyzer, complementary DNA (cDNA) was synthesized and amplified by PCR as previously described [7]. Primers (synthesized by Qiagen) and annealing temperatures are described in Table 1. Equal loading of template was confirmed by co-amplifying the housekeepr gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH). PCR products were separated, visualized, and photographed as previously described [6,7].

Statistical analysis. The indicated datasets were individually compared as described in the figure legend by the paired Student's *t* test using the Graph Pad Prism 3.0 program (Graph Pad Software, San Diego, CA).

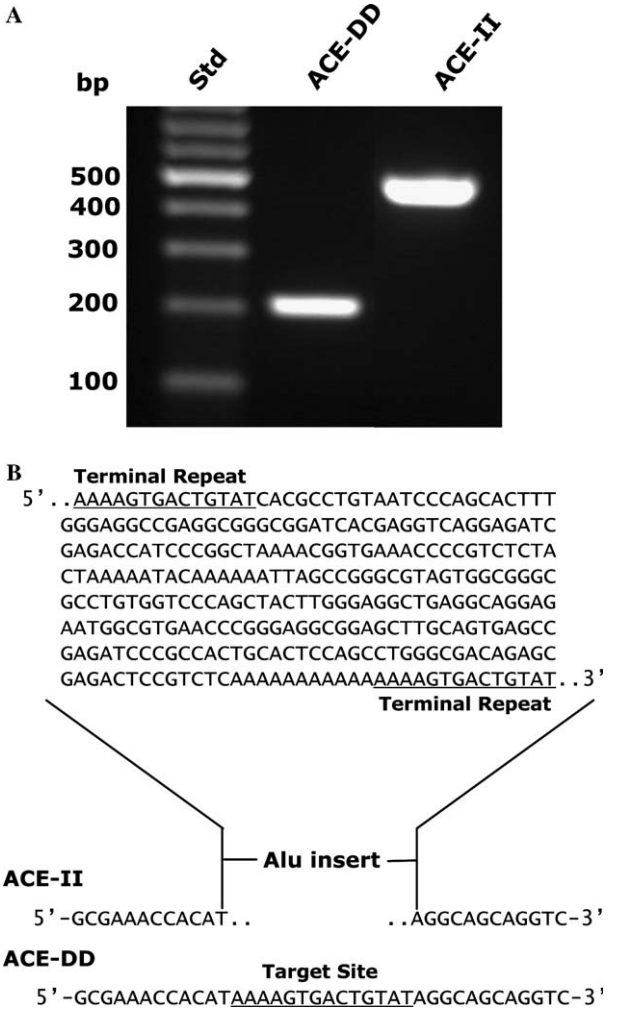

Fig. 1. PCR and sequence analysis of the ACE polymorphic Alu insertion. (A) PCR analysis of the ACE polymorphism in primary cultures of human endothelial cells for the presence of the Alu repeat. Genomic DNA was extracted, PCR-amplified, and electrophoresed on 2% agarose gels as previously described [6]. Migration of the molecular weight (MW) standards is indicated as base pairs (bp). DNA bands in the higher MW range indicate the presence of an Alu insert, those in the lower range, its absence. (B) PCR products were purified and sequenced. Shown are aligned sequences from the region surrounding the unoccupied (ACE-DD) and occupied (ACE-II) target site in the ACE gene. Terminal repeats and target site are underlined.

Table 1
RT-PCR primers and annealing temperatures

Gene	5′-flanking primer	3′-flanking primer	Annealing temperature (°C)
uPA receptor	CCTGCGGTGCATGCAGTGTAAGACCAA	ACAGCCACGGAGGTGGCGGTCATCC	55
uPA	TGGCACAAGCTGTGAGATCACTGG	GTCGTTCACCCTGGTGGTGCCACAG	55
Numb	GGCCTCAGCTGCTCCTCTGCAGCCAG	ACCTCTGTATGCCTGTCTGCTGAGGCC	55
Klotho	ACAGCATCAGGCGTGGACTCTTC	CTCGCTGGCCATGCAGCGATAGTACTG	55
Nanog	TACCTCAGCCTCCAGCAGATGCAAGAACTCTC	GTTCCAGGACTGAGCGGTCCAGG	55
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	62

Results and discussion

In this study, the genotypes of various EC were determined (Fig. 1) and matched for gender, age, and tissue source. ACE-II and ACE-DD cell survival was assessed after slow starvation. Traditionally, starvation involves the abrupt withdrawal of serum, which induces apoptosis. We modified this assay by allowing cells to slowly exhaust available nutrients. We believe this approximates progressive disease states found in old age, allowing cells to mount a survival response [7]. We also exposed cells to captopril for 2 weeks, followed by slow starvation. We show dramatic survival of ACE-II cells as compared to ACE-DD (Fig. 2). Captopril significantly enhanced ACE-DD viability but had little effect on the already-viable ACE-II cells (Fig. 2).

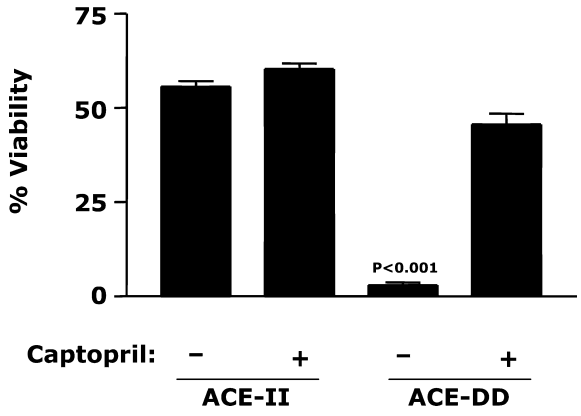

Fig. 2. Differential cell survival after slow starvation. Confluent cultures of ACE-II and ACE-DD human endothelial cells were incubated for 2 weeks with vehicle or 1 mM captopril. Medium was changed two-to-three times per week. This was followed by slow starvation until the onset of apoptosis as previously described [7]. Viability was assessed from the trypsinized monolayer combined with the culture medium containing floating dead cells by trypan blue exclusion. Bars represent means ± SEM of at least three individual experiments. $P < 0.001$ vs. non-treated ACE-II cells or captopril-treated ACE-DD cells.

ACE cleaves angiotensin-I (AngI) to yield angiotensin-II (AngII), a peptide with well-known pro-apoptotic effects [8]. We investigated whether ACE-DD and ACE-II cells had different levels of AngI and AngII. ACE-II cells had low levels of AngII and high levels of AngI, indicating a lack of conversion of AngI to AngII (Fig. 3A). ACE-DD cells had high levels of AngII and correspondingly low levels of AngI (Fig. 3B), suggesting high ACE activity. These data explain why ACE inhibition by captopril rescued ACE-DD cells from slow starvation but had minimal effect on ACE-II cells, which already had low AngII levels and higher survival. Non-human cells are, by default, ACE-DD [9], which explains why pharmacological ACE inhibition enhanced bovine EC survival [7]. The differential expression of AngII between the genotypes and the exclusive responsiveness of ACE-DD (human and non-human) EC to captopril validates the polymorphism's effect on cell survival through the renin–angiotensin system.

We observed that ACE-II cells grew better than ACE-DD, (61% vs. 44% viability, respectively). In fact, we have continuously passaged an ACE-II primary EC culture for almost 5 years, suggesting it may have undergone spontaneous immortalization. Pharmacological ACE inhibition enhances cell survival by upregulating genes involved in cell growth, survival, and immortalization [7]. Here we analyzed their expression in ACE-II and ACE-DD cells, together with genes involved in cellular self-renewal.

Urokinase (uPA) has been implicated in cell survival. It mediates the anti-apoptotic activity of TGFβ in vascular smooth muscle [10] and protects neurons from β-amyloid toxicity [11]. Historically, uPA has been described as a protease; however, its anti-apoptotic effect is mediated by its receptor uPAR [12,13]. Here we show that uPA expression is increased in ACE-II cells as

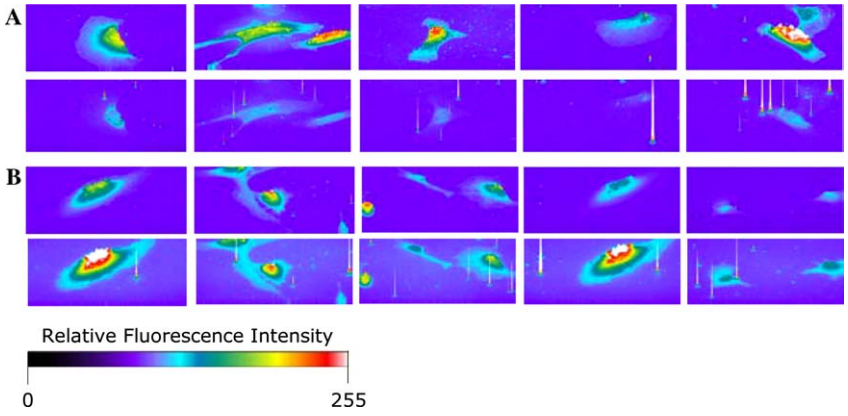

Fig. 3. AngI and AngII levels in ACE-II and ACE-DD cells. Dual immunofluorescent analysis was conducted as previously described [7] on non-confluent monolayers of ACE-II (A) and ACE-DD (B) endothelial cells. Both panels show the intensity profiles from five randomly selected fields for each genotype. In each panel, the top row illustrates AngI staining whereas the bottom row represents AngII staining for the same field. The pseudo-color scale corresponds to the levels of fluorescence intensity from 0 to 255. Magnification: 400×.

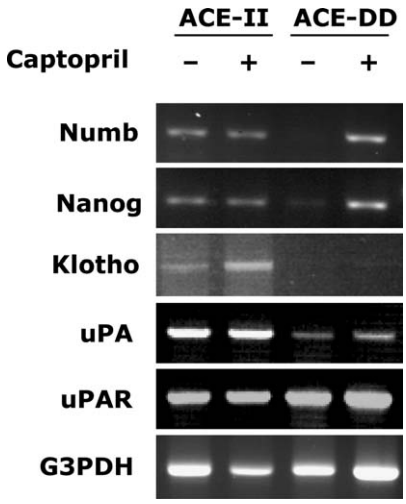

Fig. 4. Differential gene expression in ACE-II and ACE-DD cells. Confluent endothelial cell cultures were treated for 2 weeks with vehicle or 1 mM captopril, followed by slow starvation until the onset of apoptosis. RNA was extracted at the conclusion of the experiments and evaluated with the Agilent 2100 Bioanalyzer for quantity and quality. RT-PCR was performed as previously described [7]. G3PDH was used to control for loading differences.

compared to ACE-DD whereas the uPAR levels remain unchanged (Fig. 4).

Maintenance of pluripotency in stem cells involves various homeotic genes. *Numb*, a member of the notch pathway, plays a critical role in the maintenance of neural progenitor cells [14]. It also participates in their asymmetric cell division [15]. Little work has been done on the role of *numb* in the vasculature, even though it is expressed in adult lung EC [16]. Here we show that *numb* is exclusively expressed in ACE-II cells. Upon captopril treatment, however, ACE-DD cells upregulate *numb* (Fig. 4).

Nanog, another homeodomain protein, is present only in embryonic stem cells (ESC) and is absent from differentiated cells. It directs the self-renewal of ESC and is critical in the maintenance of pluripotency [17]. Sustained expression of *nanog* is attained through the wnt pathway [18]. We are the first to report that *nanog* is expressed in endothelial cells, with much higher levels in ACE-II cells (Fig. 4). Captopril treatment of ACE-DD cells induced *nanog* expression, suggesting that it can be regulated through the renin–angiotensin system. The accepted view is that EC cultures consist of differentiated cells; however, these data suggest that they may contain a fraction of precursor cells. In a novel angiogenic assay [19], captopril treatment enhances the secondary-sprouting capability of EC [7], a process that involves the spontaneous survival and expansion of EC precursors [20].

Klotho is a key regulator of cellular senescence and aging. Its role involves ion and vitamin D homeostasis; however, its function has not been completely elucidated [21]. Interestingly, *klotho* is expressed in ischemic EC in vivo and may regulate angiogenic processes [22]. Most significantly, *klotho* expression is controlled by AngII. Long-term infusion of AngII in rats downregulated *klotho* mRNA and protein. Furthermore, *klotho* overexpression prevented AngII-mediated renal damage [23]. We found that *klotho* is exclusively expressed in ACE-II cells and is upregulated by captopril. ACE inhibition did not induce *klotho* in ACE-DD cells (Fig. 4), possibly due to their sustained expression of AngII (Fig. 3).

Various human diseases, such as heart and kidney, show increased ACE activity and higher levels of AngII. Conversely, captopril administration is protective [24,25]. Only ACE-DD individuals were helped by captopril but the exact mechanism remained unclear [26]. We have shown that ACE-DD cells had higher levels of AngII and were more prone to cell death than ACE-II cells (Figs. 2 and 3). However, captopril rescued ACE-DD cells (Fig. 2). These results suggest that the protective effect of ACE inhibition occurs at the cellular level through the upregulation of genes involved in cell survival and renewal.

As previously mentioned, the ACE-II genotype is more frequent in extreme athletes [2]. One would expect athletes to survive well into old age; however, the ACE-II variant is less frequent in the elderly [3]. A potential clue was derived from a cohort study showing that the ACE-II genotype correlated with higher incidence of breast cancer [27]. Our results suggest that ACE-II cells have a survival advantage during stress (Fig. 2). Stressful conditions are often found in tumors. In addition, ACE-II EC would also have increased survival leading to robust vasculature. This would benefit extreme athletes but would be detrimental to cancer patients by increasing tumor angiogenesis. In another example, the ACE-II genotype increased the risk for Alzheimer's disease (AD) [28] but was protective for AMD [6], both diseases of age with similar degenerative pathologies. We propose that, depending on which cells survive, disease processes will either be aborted or advanced. AD involves neuronal cell death by activated glia whereas AMD entails RPE degeneration. We hypothesize that glial cell survival by ACE inhibition in AD leads to gliosis resulting in increased neuronal cell death, but in AMD, RPE cell survival leads to the attenuation of disease.

With over a million Alu elements in the human genome, their functional roles have been intriguing. Some have described them as "junk" DNA while others consider them a genuine system equivalent to bacterial plasmids or even as disposable genes [29]. It appears that Alu elements have played a role in human evolution because Alu invasions were synchronous with diverging lines of primates [9]. They have also contributed to disease [30] by inserting in coding regions, upsetting gene structure. Since coding sequences are less abundant, Alus litter the landscape of non-coding DNA. This contributed to their recruitment for regulatory functions

as in the case for the ACE Alu. In this paper we show that this controversial polymorphism directly impacts cell survival under stressful conditions. By demonstrating a functional role for the ACE Alu, we hope to provide a powerful tool to explain its association with human disease and biology.

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