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INHIBITION OF ENDOTHELIN-1 INDUCED MYOCARDIAL PROTEIN SYNTHESIS BY AN ANTISENSE OLIGONUCLEOTIDE AGAINST THE EARLY GROWTH RESPONSE GENE-1

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We explored the role of the recently discovered "early growth response gene-1 (Egr-1)" in the induction of myocardial protein synthesis by endothelin-1.
Endothelin-1 stimulated protein synthesis (i.e. ^{3}H-phenylalanine incorporation) in isolated adult rat cardiomyocytes more than 2-fold. Addition of a 15mer Egr-1 antisense oligodeoxyribonucleotide complementary to the first 5 codons of the Egr-1 mRNA completely blocked endothelin-induced protein synthesis. A single base mismatch in the oligonucleotide sequence abolished the inhibitory effect. T_3-induced stimulation of protein synthesis was unaffected by the antisense oligonucleotide.
These results indicate that the Egr-1 gene product is involved (putatively as a third messenger) in the signal transduction cascade initiated by endothelin-1 which eventually culminates in the induction of cardiac protein synthesis.

Hypertension-related cardiac hypertrophy is of medical importance because it carries an increased risk of cardiac death and pump failure (1). Endocrine or paracrine factors including α_1-adrenergic stimuli (2), angiotensin II (3), and endothelin (4,5) have been shown to induce hypertrophy and/or remodeling in isolated neonatal cardiomyocytes from rat (2,4,5) or chick (3). Induction of protooncogenes and immediate/early genes by endothelin-1 has also been demonstrated (4,5). However, it is unknown which of these genes act as transcription factors ("third messengers") in the paracrine induction of myocardial growth.

Egr-1 [also known as Zif 268, Krox 24, NGFI-A (6), and Tis-8, review and extensive references in 7] is a member of a family of

Abbreviations
Egr-1 Early growth response gene 1. EGR (capital letters) refers to the human homologue (7).

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recently discovered zinc-finger containing transcription factors (Egr-1 and the human genes EGR-2, 3 and 4) that have been isolated using differential cloning techniques of stimulated vs. quiescent cells in the presence of cycloheximide (7). They belong to a group of immediate/early genes expressed during induction of cellular growth and/or cell division. They are assumed to take part in the signal transduction cascade from diverse stimuli to genes that are typical of the differentiated state of the cell (7). The induction kinetics resemble those of c-fos and repression of Egr-1 transcription by c-fos has recently been demonstrated as a possible link between these two transcription factors (8). We therefore hypothesized that Egr-1 may have a pivotal role in the induction of myocardial growth by hormones and/or paracrine substances.

We chose to study adult cardiomyocytes because of our interest in the mechanisms of cardiac hypertrophy in the adult and because of the purity of the adult cardiomyocyte preparation which is virtually free of fibroblasts (9). Endothelin-1 was investigated because of the proximity of the capillary endothelium to the cardiomyocyte in the heart and recent suggestions of a role for endothelin in the development of hypertension (10) and as a growth factor in mesangial cells (11).

MATERIAL AND METHODS

An unmodified antisense oligodeoxyribonucleotide to the first 5 base triplets of the Egr-1 coding region was synthesized using the sequence for rat Egr-1 (NGFI-A) in (6). The ATG codon suggested by Sukhatme (12) was taken as the start site; originally, Milbrandt had proposed an ATG 84 nucleotides downstream from this site (6). Cardiomyocytes were isolated from the heart of Wistar-Kyoto rats (200-250g) by the collagenase method (9) and used 60-90 min after preparation. 10^5 cells in medium BM 86 were attached (60 min, 37°C) to a 10 cm^2 Petri dish precoated with laminin (1 $\mu g/cm^2$). Contamination by non-myocytes was less than 0.5% as assessed by immunofluorescence staining with an antibody to troponin T. 1.5μM unmodified oligodeoxynucleotide (synthesized on a Pharmacia Gene Assembler and purified over a NAP 10 column) were added. Cells were kept at 37°C in a CO_2-incubator for 18 hrs. Endothelin-1 (10^{-7}M; Peninsula, England) or buffer (control) was added 5 min after ^{3}H-phenylalanine (Phe, 1μCi) and incubation continued for 3hrs. Cells were then precipitated and washed 3x with 4% perchloric acid, the pellet resuspended in 1N NaOH and counted (13). Preincubation with T_3 (3',3',5-triiodo-L-thyronine; 0.8 nM) was for 24 hours to allow for the slower induction of protein synthesis by T_3. ^{3}H-Phe was added for 3 hours and acid precipitation performed as above. ^{3}H-Phe transport into the cells was assessed in the presence of $3x10^{-5}$M cycloheximide. Total RNA was isolated by the Chirgwin method (14) and a 2.1 kb fragment of Egr-1 (OC68 insert, 12) not overlapping the first 5 codons of the coding region was used as a probe. Labeling was performed by the random oligo-

nucleotide method. A 0.77 kb probe for total actin was purchased from Dianova, Hamburg. Statistical evaluation was by ANOVA. All experimental procedures involving animals conformed to the guiding principles of the American Physiological Society.

RESULTS

Fig. 1a shows that the antisense sequence completely inhibited endothelin-induced protein synthesis. Basal synthesis and cell morphology were unaffected indicating that the antisense oligonucleotide was not toxic. Introduction of a single mismatch at position 8 of the 15mer abolished the inhibitory effect of the antisense sequence; the same was true for an oligonucleotide containing two mismatches at positions 4 and 10 (Fig. 1a).

The well-known induction of myocardial protein synthesis by T_3 (15) was not affected by the antisense oligonucleotide (Fig. 1b). This demonstrates that Egr-1 has no direct role in the T_3 signal transduction pathway and excludes unspecific toxicity of the antisense sequence. To exclude that endothelin and T_3 increased ^{3}H-phenylalanine (^{3}H-Phe) transport into the cell thus altering the specific activity of the intracellular isotope, ^{3}H-Phe transport

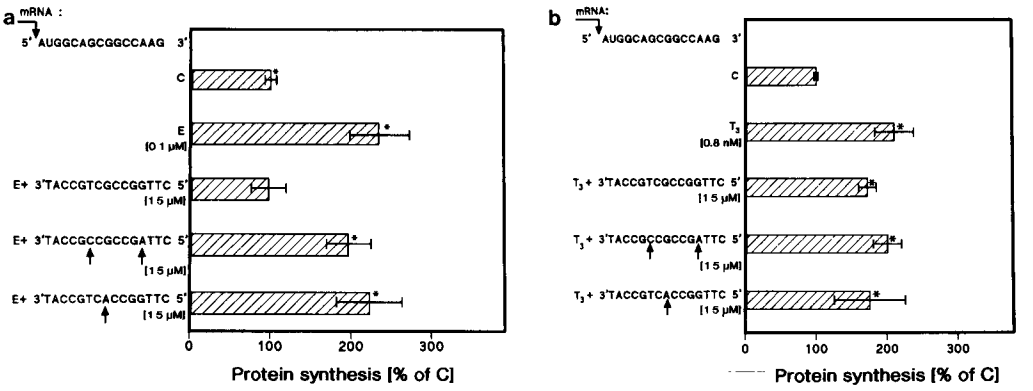
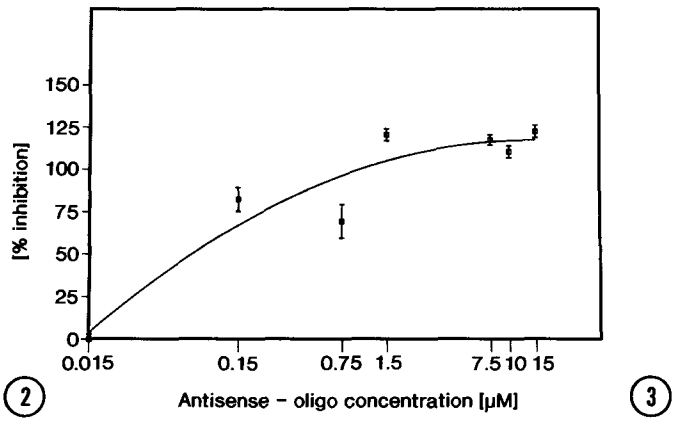


FIG. 1. Effect of Egr-1 antisense oligonucleotides on protein synthesis.(a) endothelin-1 (E, 10^{-7}M) stimulated protein synthesis more than 2-fold (C=control without endothelin). The antisense oligodeoxynucleotide complementary to the first 15 bases of the Egr-1 mRNA coding region completely abolished this stimulation (third column from top). A single or double mismatch in the antisense sequence (indicated by arrows) resulted in an ineffective oligonucleotide. Note inversion of the antisense sequences (3'- 5') on the vertical axis to facilitate sequence comparison. n=31 independent experiments (in duplicate) for C (=Control, no hormone) and endothelin, n=6 for antisense and mismatched oligos. Mean ± SEM, *p<0.01.

(b) T_3-induced stimulation (0.8 nM) of protein synthesis was unaffected by the antisense oligonucleotides. C=control, n=5 independent experiments in duplicate for each column, *p<0.01 compared to control. Differences between columns 2 to 5 not significant. Antisense sequences as in a.



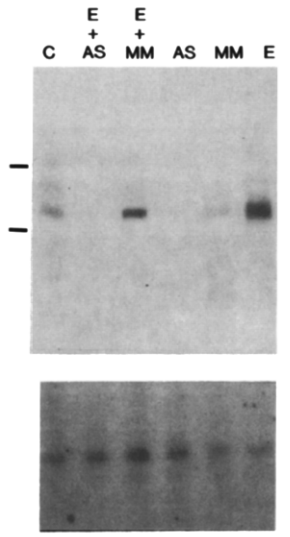


FIG. 2. Dose-response curve for the effect of the antisense oligonucleotide on the induction of protein synthesis by endothelin. Experimental details were as in FIG. 1.

FIG. 3. Effect of the Egr-1 antisense oligodeoxynucleotides on the level of Egr-1 mRNA (Northern blot). C: Control, no addition. E: Endothelin-1, 10^{-7}M, 30 min. AS: Antisense sequence, fully matched, 1.5μM, 18hrs preincubation. MM: Antisense oligo, mismatched at positions 4 and 10. Lower part: actin controls (Loading in lanes 5 and 6 is somewhat lower; the effect of endothelin is therefore even more marked than the 10-15 fold induction shown in E vs. C).

was studied. No influence of the oligonucleotides was observed (0.299 ± 0.076, 0.287 ± 0.089, 0.284 ± 0.210 nmol Phe/10^5cells/3hrs for control, endothelin, and T_3, respectively, differences not significant, 4 experiments in duplicate). A minimum of 6 hours was required for uptake of the antisense oligonucleotides into the cells, but more consistent results were obtained after 18 hours preincubation (data not shown). This time course is in agreement with other work using antisense oligodeoxynucleotides (16,17). There was a dose-response relationship with an IC_{50} of approximately 0.075 μM for the antisense oligonucleotide (Fig. 2). Complete inhibition of stimulated protein synthesis was seen at concentrations above 0.75 μM. The antisense oligonucleotide dramatically reduced the level of Egr-1 mRNA after incubation with endothelin-1 (Fig. 3), suggesting degradation of the message as the mechanism of action of the antisense oligonucleotide.

DISCUSSION

Our results demonstrate that an antisense oligodeoxyribonucleotide directed against the early growth response gene-1 (Egr-1)

inhibits stimulation of myocardial protein synthesis by endothelin-1 but not by thyroid hormone. The oligonucleotide acts by decreasing the cellular level of Egr-1 mRNA. This establishes induction of the transcription factor Egr-1 as an obligatory step in the cascade leading from membrane binding of endothelin to the induction of hypertrophic growth and/or remodeling in adult cardiac myocytes. Egr-1 may therefore be considered as a third messenger step for the action of endothelin in this system. Formally, it cannot be excluded that the Egr-1 gene product acts by some more general mechanism, eg. by stimulating synthesis of tRNA or induction of the RNA polymerase. However, this seems very unlikely since neither basal protein synthesis (i.e. in the absence of endothelin) nor the hypertrophic effect of thyroid hormone were inhibited by the antisense oligonucleotide.

Therefore, the most likely interpretation of our results is that the Egr-1 gene product acts as a transcription factor ("third messenger") in a cascade leading to the induction of a gene program characteristic for endothelin-1. Diacylglycerol is the likely second messenger (Neyses et al, unpublished). This interpretation is further strengthened by the recent finding of Gupta et al (18) that overexpression of Egr-1 in neonatal rat cardiomyocytes regulates transcription of the α-myosin heavy chain gene. To the extent that similar results can be obtained in other cell systems, Egr-1 may well prove to have a role as "master switch" similar to the fos/jun complex (19) in signal transduction pathways from the membrane to the nucleus.

The ease and specificity of this approach along with the low concentrations of antisense oligonucleotides required (an order of magnitude less than in other cell systems, 17) suggest that the antisense procedure may be suitable for investigating the role of other genes in isolated cardiomyocytes. It may be particularly useful in adult cardiomyocytes because transfection of these cells in numbers sufficient for biochemical analysis has proven difficult. The likely mechanism for the reduction of the Egr-1 message is accelerated degradation of mRNA by RNAse H as has been demonstrated in Xenopus oocytes (20). Inhibition of translation seems less likely since in this case the level of mRNA would not be reduced (17). In more general terms, inhibition of single genes in the myocardium may prove to be a route towards gene therapy in cardiac disease if organ targeting can be achieved, eg. by liposomes (17).

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