Regulated expression of a foreign gene targeted to the ischaemic myocardium

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Abstract

Objectives: Regulated expression of transferred foreign genes may be an important feature of gene therapy. Because coronary artery disease often involves intermittent myocardial ischaemia followed by periods of normal cardiac function it will probably be necessary to regulate the expression of putative therapeutic/cardioprotective genes directly in response to ischaemia-associated signals. The objectives of the current study were to develop a combination of gene regulatory components that can be used to target a product to the myocardium and limit the expression of the gene to periods of ischaemic activity. Methods: Expression plasmids were constructed containing muscle-specific promoters and hypoxia-responsive enhancer elements linked to a reporter gene. The regulation of these constructs by hypoxia or experimental ischaemia was measured following transient expression in cultured cells or after direct injection of DNA into the rabbit myocardium. Results: A single set of hypoxia response elements placed immediately upstream of the minimal muscle-specific α-myosin heavy chain promoter conferred potent positive regulation of this promoter by hypoxia in vitro and by ischaemia in vivo. Induction by ischaemia persisted for at least 4 h and returned to the baseline level within 8 h. Conclusions: Hypoxia responsive regulatory elements, in combination with weak tissue-restricted promoters incorporated into an appropriate vector system may allow controlled expression of a therapeutic gene in ischaemic myocardium. © 1997 Elsevier Science B.V.

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1. Introduction

A number of procedures have been described for the efficient delivery and expression of foreign genes in the heart and coronary vasculature. Replication-defective adenoviral vectors [1–7], adeno-associated viral vectors [8], and disabled herpes virus vectors [9] can result in highly efficient transfer to rat, dog, and pig cardiac myocytes in vivo. Naked DNA and liposome complexes have also provided substantial, although perhaps more localized, delivery to cardiac myocytes in vivo and in vitro [10–14]. In both cases either direct intramuscular injection or infusion into the coronary artery with or without the assistance of a catheter appears to be quite effective in transferring significant amounts of transcriptionally active genetic material into both the vascular cells and the cardiac myocytes. Although most of the studies to date have analyzed the expression of transferred reporter genes, biologically relevant genes have also been transferred to both the vascular tissue and myocardial tissue [15–20].

Existing vector systems can provide adequate delivery of a gene, and it seems likely that immunogenic responses will eventually be reduced or even eliminated [8,21]. How-
ever, appropriate regulation of the therapeutic gene after delivery remains a problem. Localized injections, or the use of tissue-specific cell surface recognition molecules can afford one level of vector targeting, reviewed in [10,11], and the use of tissue-specific promoters (e.g. von Willebrand factor [22], α-myosin heavy chain [3,23], myosin light chain-2 [24]) can provide another degree of localized expression. Unfortunately, these elements generally direct constitutive expression in the target cell or tissue that is unrelated to the (pathophysiological) cellular environment. A third possible control manoeuvre involves the inclusion of an externally regulatable promoter element, such as a hormone-, antibiotic-, or metal-responsive enhancer, in the therapeutic gene promoter [25–27]. In this case the expression of the targeted gene can be modulated by treatment or withdrawal of a specific effector (e.g. hormone, antibiotic, metal). While this clearly may be advantageous in some circumstances, such an approach will require additional monitoring and intervention. It would be preferable for expression of the targeted gene to be directly responsive to a component of the disease phenotype.

Our understanding of the pathophysiology of coronary artery disease and myocardial ischaemia is incomplete, but there is substantial evidence that myocardial cell damage occurs during both the ischaemic episode and the subsequent reperfusion [28–35]. Ischaemic hearts experience regional cycles of hypoxic and oxidative stress that punctuate normal cardiac function, resulting in progressive cell loss and tissue damage. Animal studies have identified a number of interventions that can reduce the damage caused by ischaemia and reperfusion, including overexpression of heat shock proteins [36,37], infusion of anti-inflammatory agents [35,38], treatments with tissue plasminogen activator, vasodilators, and antioxidants (reviewed in [39,40]), and overexpression of anti-apoptotic genes such as Bcl2 [41,42]. These agents are all candidates for gene therapy that could afford significant cardioprotection during ischaemic episodes if their delivery and activity were appropriately regulated. Appropriate regulation in this case should involve elevated activity of the agent during heightened periods of ischaemic activity, and quenched levels when the ischaemia subsides. This may be possible if the transferred gene is under the control of elements that respond directly to a component of ischaemic or oxidative stress.

In the present study we have examined the possibility that hypoxia response elements (HREs) incorporated into a minimal muscle specific promoter can be used to regulate the expression of a test gene (firefly luciferase) by hypoxia and ischaemia using cultured cardiac myocytes in hypoxic culture and a rabbit model of experimental myocardial ischaemia. We show that a cartridge containing multiple copies of a hypoxia response element from the human erythropoietin gene placed upstream of a minimal α-myosin heavy chain (MHC) promoter, is tightly regulated by hypoxia in vitro and by ischaemia in vivo. The results indicate that this combination of regulatory elements may be useful in the design of vectors for the targeting and regulated expression of a therapeutic gene in the ischaemic myocardium.

2. Methods

2.1. Plasmid constructions

pGEM4-HRE, containing four copies of the erythropoietin HRE cloned into the BamHI site of pGEM4 (Promega Biotech, Madison, WI), was a gift from Greg Semenza (Johns Hopkins University, Baltimore, MD) [43]. The α-MHC promoter constructs truncated to −1.2 kb and −86 bp were generous gifts from Tom Gustafson [44] and Bruce Markham [45] respectively, pGL (Gene Light) vectors were obtained from Promega, pSVHRE was made by inserting a 240 bp HindIII PCR fragment from pGEM4-HRE into the multiple cloning site of pGLBP which contains the proximal SV40 promoter linked to the luciferase gene (Promega). To insert the α-MHC promoter fragments the SV40 promoter was removed from pSVHRE, and replaced with the 1.2 kb or 86 bp fragments of the α-MHC promoter to create pα-MHC1.2HRE, and pα-MHC86HRE. Internal controls pRSV-luciferase and pCAT3PV (SV40 promoter linked to the Escherichia coli chloramphenicol acetyltransferase (CAT) gene), were from Promega Biotech.

2.2. Cell culture

The isolation of cardiac myocytes from neonatal rat hearts has been described in detail elsewhere [46,47]. Briefly, hearts from 30–40 pups were minced and subjected to serial trypsin digestion to release single cells. After the final digestion the cells were washed and preplated for 0.5 h in MEM with 5% fetal calf serum. Non-attached cells were replated in 60-mm Falcon dishes at $4 \times 10^6$ cells per plate in the same medium with 0.1 mM bromodeoxyuridine (BrdU) for 3 days after which time the BrDU was removed. Cultures were transfected on day 1 after isolation and used for experiments after a further 3 or 4 days. The preplating and BrDU steps reduce the background of non-myocardial cells. These procedures reproducibly generate cultures that contract synchronously at >250 beats per minute consistent with the normal beating rate of the intact rat heart [48]. Cultures were maintained in humidified air with 5% CO$_2$ at 37°C [46,47] except where indicated. Culture of HeLa cells and C$_2$C$_2$ skeletal myoblasts have been described previously [49–51]. Calcium phosphate transfections, luciferase and CAT quantitation, and protein determinations in cell and tissue extracts were performed as described previously [22,49,50,52,53].
2.3. Hypoxia

Transfected cultures were maintained in MEM with 5% fetal calf serum supplemented with 3 g/l glucose until exposure to hypoxia (pO₂ = 8–12 mmHg). Details of our methods for exposing cells to hypoxia have been described previously [47,54]. Oxygen was continuously monitored with an oxygen electrode (Controls Katharobic, Philadelphia, PA) inside the chamber and contractility was monitored by edge detection as described previously [47,54]. After 16 h of hypoxia, plates were harvested and lysed for protein and luciferase assays. Under these conditions, cardiac myocytes retained contractility for the duration of all experiments, and intracellular ATP and medium glucose levels were maintained throughout as previously described (data not shown, see references [47,54,55]).

2.4. Gene transfer and ischaemia/reperfusion in rabbit heart

Male New Zealand White rabbits (2.5–3.0 kg) were premedicated with intramuscular Hypnorm, Jansen Pharmaceuticals [0.3 ml/kg; fluanisone (10 mg/ml), fentanyl citrate (0.315 mg/ml)] and treated with intravenous midazolam (0.25–0.5 mg/kg) to permit endotracheal intubation (size 3–4). Animals were ventilated (0.3–0.4 l/min/kg) on a small animal ventilator with 1–2 cm H₂O of positive end-expiratory pressure. Animals were maintained under anaesthesia with an inhaled mixture of equal concentrations of nitrous oxide and oxygen plus 1% halothane at a flow rate of 2 l/min. The chest was opened by a left thoracotomy, and the pericardium removed for DNA injection. DNA was introduced by injection in a Hamilton syringe; 50 µg SV40-CAT plasmid and 50 µg of α-MHC<sub>36</sub> ± HRE plasmid suspended in phosphate-buffered saline, firstly into the cardiac apex (three injections) and secondly into the proximal left ventricular wall (three injections). Postoperative analgesia was administered (0.2 mg/kg buprenorphine i.m.). Seven days after DNA injection rabbits were subjected to a second thoracotomy for implementation of transient myocardial ischaemia as described previously [56]. Premedication, ventilation and anaesthesia were performed as described above. After thoracotomy, the 1st obtuse marginal artery was ligated by insertion of a suture at the midpoint between the atrioventricular groove and the cardiac apex. The presence of an area of ischaemia was confirmed by visualisation of tissue discoloration and by ECG changes as described previously [56]. After 15 min of ischaemia the suture was removed to allow the heart to be reperfused. Intravenous quinidine was administered 5 min before ligation to decrease the likelihood of ischaemia or reperfusion-induced arrhythmia. Ventricular arrhythmias during surgery were treated with an 8 joule epicardial DC shock. Post-operative analgesia was administered as described above. Sham-operated animals were subjected to the same procedures but without coronary ligation. One to eight hours after surgery animals were sacrificed with a lethal dose of pentobarbital sodium and the heart excised for assays of reporter gene expression [22]. All procedures were performed under license in accordance with NIH guidelines or in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986.

3. Results

To characterise the expression and hypoxia response of the promoter elements, the different plasmids were trans-
fected into muscle and non-muscle cells and assayed for the transient expression of luciferase under aerobic and hypoxic conditions as described in Section 2. Luciferase activities are expressed relative to the aerobic activity of the RSV plasmid (Fig. 1a) or the SVHRE plasmid (Fig. 1b), arbitrarily designated as 1 for each cell type. The results from control plasmids, without HREs, are shown in Fig. 1a. The RSV promoter construct was expressed at high levels in all cell types and was induced slightly during exposure to hypoxia. The other control plasmids, pGLSV2 and α-MHC, expressed at a fraction of the RSV levels, as expected for truncated promoters [44,45] and were also induced slightly (<2-fold) by hypoxia. The molecular mechanism(s) for the small inductions of these diverse promoters by hypoxia is not clear although it may be related to the presence of hypoxia responsive promoter elements. We have previously described activation of transcription factor AP1 by hypoxia, and the transcription factor Sp1 may also be sensitive to hypoxia ([52,54], and Webster et al. unpublished observations). The α-MHC construct expressed preferentially in muscle cells, in agreement with previous reports [45], and the expression increased by 1.4 ± 0.3-fold (n = 3) under hypoxia.

The hypoxic induction of the HRE-containing promoters was enhanced compared with the parent plasmid (minus HRE) in all cases (Fig. 1b). The non-tissue selective SV40-HRE promoter was expressed at high levels in all cells tested and was induced 4- to 6-fold by hypoxic incubation in all cell types (V columns in Fig. 1b). In agreement with previous reports, the level of expression of the α-MHC promoters was muscle specific [23,44,45]. The −1.2 kb α-MHC promoter (labeled α2 in Fig. 1b) directed expression poorly in HeLa and C2C12 cells but was stronger in cardiac myocytes. This result is consistent with repressor activities within the −1.2 kb region that may reduce expression in non-cardiac cells including skeletal muscle [57], and is also consistent with the thyroid hormone requirements for high level expression of this promoter [23,45]. The 1.2 kb α-MHC construct was expressed at relatively higher levels in aerobic cardiac myocytes and was induced by about 4-fold under hypoxia, similar to the SV40-HRE promoter. The smaller α-MHC-HRE construct (labeled α1 in the figure), truncated to −86, contains the minimal elements that retain muscle specificity [45]. This construct was active in both C2C12 myocytes and cardiac myocytes but the expression was significantly lower than

![Graph](https://via.placeholder.com/150)

**Fig. 2. Induction of pα-MHC₈₆-HRE in ischaemic rabbit hearts.** The surgical procedures, injections, and assays are described in Section 2. One week after the first thoracotomy and injection of DNA, a second thoracotomy was performed and the animals were subjected to ischaemia by left marginal artery ligation as described or to sham operation only. Fifteen minutes after the ligation the suture was removed and the hearts were reperfused for varying times as indicated on the figure. At each time point rabbits were sacrificed and the hearts were removed, the ventricles and ischaemic zones were dissected, and the appropriate regions of tissue were lysed and analysed for reporter gene expression as described. The results represent data from a minimum of 3 separate experiments in each case with S.E.M. as indicated. The 1 h time point for pα-MHC₈₆-HRE includes results from 5 experiments, and the 4 h time point includes data from 10 experiments. Induction of luciferase gene expression by ischaemia at the 1 and 4 h time points was significantly different from the shams with p < 0.001 at both time points by t-test, comparing related samples.
the other two test plasmids in aerobic cardiac myocytes. This is also consistent with previous reports that have described multiple elements upstream of −86 that are required for high level cardiac specific expression of the α-MHC promoter [23,45]. Both of the α-MHC promoter-HRE constructs were induced by hypoxia, but fold induction was higher (9.2 ± 1.2) for the −86 bp promoter. This effect may be related to the low aerobic expression of this promoter in the cardiac cells or to the closer proximity of the HRE elements to the transcription initiation site compared with the 1.2 kb promoter.

These in vitro results confirm the tissue specificity, hypoxia inducibility, and relative expression of the α-MHC-HRE promoter constructs in skeletal and cardiac myocytes in vitro. The −86 α-MHC-HRE construct was selected for analyses in vivo because it had lower basal expression in aerobic cardiac myocytes and showed the highest relative induction by hypoxia. In vivo expression studies were carried out after intramuscular injection of control (RSV-CAT, SV2CAT, or GL2PV) and test plasmids as described in Section 2. Repeat thoracotomies for ischaemia or sham procedures were performed one week after the initial injections to allow recovery and stabilization of the tissue. An ischaemic interval was imposed for 15 min as described in Section 2, after which time the hearts were reperfused for varying times before sacrificing the animals and removing tissue for reporter assays as described above. The results of these assays are shown in Fig. 2.

For all data points the expression of the test promoter (α-MHC−86) was normalised by reference to the internal control (SV40) and each time point represents the relative expression of luciferase in the ischaemic hearts compared to the sham treatments after normalization. One hour after the ischaemia, expression of the luciferase reporter gene in pα-MHC−86/HRE injected hearts was increased by 4.05 ± 1.0-fold (n = 5) compared with the sham. Four hours post ischaemia luciferase expression was 4.6 ± 0.74-fold (n = 10) higher than the sham. After 8 h the luciferase expression of pα-MHC−86/HRE in the ischaemic hearts was equivalent to the sham-operated level (n = 3). There was no difference in the expression of an α-MHC50 construct lacking HREs or of the RSV-CAT construct between sham and ischaemic hearts at 4 h, indicating that the induction by ischaemia required HREs (Fig. 2). These results demonstrate that hypoxia-activated transcription and translation following experimental ischaemic episodes in the rabbit heart is initiated rapidly (within 1 h) and persists for at least 4 h. Therefore it may be possible to limit the expression of a therapeutic gene to periods of myocardial ischaemia.

4. Discussion

Our results demonstrate that four copies of the human erythropoietin HRE linked to the basal promoter of the muscle selective α-MHC gene conferred inducible expression of luciferase to the promoter by both hypoxia in vitro and by a short interval of ischaemia in vivo. The response to ischaemia was rapid, with almost maximal induction within 1 h, and enhanced expression remained for at least 4 h after the ischaemic episode. The promoter activation is consistent with a direct response to hypoxia because the effect was dependent on the presence of the HRE enhancer elements. Expression returned to basal ( sham) levels after 8 h. All control plasmids including pα-MHC−86 were induced slightly by subjecting cells to hypoxia in vitro (Fig. 1a), but not by ischaemia (Fig. 2). The absence of any induction of the controls during ischaemia suggests that different elements with different response times may be responsible for these two effects. Induction of HIF-1 by hypoxia is known to occur rapidly [43,61], and probably determines the induction of pα-MHC-HRE following ischaemia. However, in our in vitro experiments, the cells were exposed to prolonged hypoxia (16 h) therefore additional factors may be activated during this period that mediate the small inductions of the control plasmids. Although we did not directly assay the cellular distribution of the injected plasmids in the rabbit heart, numerous other studies using similar protocols to ours have shown that cardiac myocytes are the major recipients of DNA transferred by this method [22,23,53,58,59]. Also the α-MHC−86 promoter retains muscle specificity ([45], and see Fig. 1), therefore it will be preferentially expressed in myocytes.

There are a number of interesting features associated with the response of the transferred promoter-HRE to ischaemia. Firstly the induction was rapid, occurring within the first hour of reperfusion (Fig. 2). The timing of the response is determined by the rate of accumulation and activity of the HRE-binding transcription factor HIF-1 [reviewed in (60)] during ischaemia and reperfusion. Previous studies indicate that this response is quite rapid [61], and our own analyses of HIF-1 accumulation in C57Bl/6 cells exposed to cycles of hypoxia and reoxygenation indicate that the binding activity appears within 20–30 min after exposure to hypoxia (data not shown). We are currently examining earlier time points following ischaemia in rabbit and rat hearts for gene expression and accumulation of HIF-1 binding activity to characterize the response time. Secondly, 15 min of ischaemia caused a level of induction of α-MHC-HRE (~5-fold) that was equivalent to the induced levels following 16 h of hypoxia in vitro (~9-fold), and the induction during reperfusion was sustained for at least 4 h. These results suggest that the HRE-mediated regulation is extremely sensitive to ischaemia in the heart; they also indicate that the heart may experience ischaemia-related alterations in gene expression for several hours after reperfusion. The duration of expression of the transferred gene after reperfusion will be determined by the stability of the DNA constructs, mRNA transcripts and encoded protein, as well as by the persistence of HIF-1 activity after reoxygenation.
These results indicate that it is possible to regulate directly the expression of a transferred gene in response to ischaemia in the rabbit heart. It may therefore be possible to regulate a therapeutic gene so that it is expressed only during episodes of ischaemia and reperfusion, so that tissue levels of the gene product will fluctuate in accordance with the duration, frequency, and severity of the ischaemia. In this study we have tested a minimally regulated promoter, but it will be possible to further manipulate and optimize the regulatory units for gene therapy purposes. In our studies a single unit of tandemly repeated copies of the HRE was placed immediately upstream of the α-MHC promoter at bp −86. This conferred an approximately 9-fold induction of expression by hypoxia in vitro. Other studies indicate that this level of induction could be increased possibly up to 40-fold by placing HRE-like elements in both 5′ and 3′ positions of the target gene [60]. Elements contained in the promoter and 3′ regions of the erythropoietin gene confer up to 1000-fold induction by hypoxia or anaemia in vivo [62].

Techniques and procedures are being developed for the successful delivery and expression of genes in the heart, and there are a number of potential therapeutic genes that may protect against ischaemic damage [35, 37–40, 63, 64]. The probability that any of these will be used for gene therapy treatment of myocardial ischaemia depends on numerous factors including reliable delivery, sustained retention, positive therapeutic efficacy, and long-term safety. The latter two issues may be related to levels of expression, regulation, and side effects. In many instances it may be undesirable to transfer a ‘therapeutic’ gene that will be expressed at high constitutive levels, that are not related to the (patho)physiological state of the target tissue. For myocardial ischaemia, the use of a weak, preferably silent promoter that is strongly inducible by ischaemia/hypoxia may allow therapeutic levels of cardioprotective agents to accumulate when symptoms are severe or frequent, and to disappear when symptoms subside. This approach provides an internal sensor to trigger an endogenous source of medication, potentially reducing the need to leave the tennis court for nitroglycerine, and averting the deleterious consequences of recurrent myocardial hypoxia and oxidative stress.

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