S100β Inhibits α1-Adrenergic Induction of the Hypertrophic Phenotype in Cardiac Myocytes*

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In an experimental rat model of myocardial infarction, surviving cardiac myocytes undergo hypertrophy in response to trophic effectors. This response involves gene reprogramming manifested by the re-expression of fetal genes, such as the previously reported isofrom switch from adult α- to embryonic β-myosin heavy chain. We now report the transient re-expression of a second fetal gene, skeletal α-actin in rat myocardium at 7 days post-infarction, and its subsequent down-regulation coincident with the delayed induction of S100β, a protein normally expressed in brain. In cultured neonatal rat cardiac myocytes, co-transfection with an S100β-expression vector inhibits a pathway associated with hypertrophy, namely, α1-adrenergic induction of β-myosin heavy chain and skeletal α-actin promoters mediated by β-protein kinase C. The induction of β-myosin heavy chain by hypoxia was similarly blocked by forced expression of S100β. Our results suggest that S100β may be an intrinsic negative regulator of the hypertrophic response of surviving cardiac myocytes post-infarction. Such negative regulators may be important in limiting the adverse consequences of unchecked hypertrophy leading to ventricular remodeling and dysfunction.

In a rat model of myocardial infarction, surviving terminally differentiated cardiac myocytes undergo a phenotypic transition post-infarction, involving cellular hypertrophy and re-expression of genes normally restricted to the fetus, including the embryonic β-myosin heavy chain (β-MHC), the skeletal isoform of α-actin (skACT), atrial natriuretic factor, and a fetal L-type calcium channel (1–4). This response is comparable to the hypertrophic response of the heart to acute pressure overload, during which the induction of several fetal genes including β-MHC and skACT has been observed (5–7). The existence of feedback mechanisms limiting the hypertrophic response is suggested by the observation that the induction of β-MHC and skACT, in response to pressure overload, is down-regulated after 8–11 days in association with moderation of the increase in myocardial mass (5, 6). Since the attenuation of the hypertrophic response post-infarction may be beneficial in tempering adverse ventricular remodeling (1), evidence for similar feedback mechanisms would be important to define the balance among opposing physiological interactions that come into play following ischemic injury.

The signaling pathways activated by myocardial infarction are likely to be multifactorial and the relative contributions of hypoxia, ischemia, and activation of local and systemic trophic factors in the hypertrophy of surviving myocytes is unknown. Hypertrophy and its associated program of fetal gene re-expression can be reproduced in vitro in cultured neonatal rat cardiac myocytes by treatment with a number of trophic effectors including peptide growth factors and α1-adrenergic agonists such as norepinephrine (NE) and phenylephrine (PE) (8–17). Studies in this cell culture model have implicated several signaling mechanisms including activation of protein kinase C (PKC) in triggering this end-response (13, 15, 17, 18). This has allowed the tracing of individual biochemical pathways that are associated with hypertrophy in myocytes, for example, the induction of β-MHC and skACT as a consequence of activation of β-PKC by α1-adrenergic agonists (9, 12, 18, 19). The focus of the current research is to identify the intermediate components of these pathways that bring about the transcription of the end-response genes (19–21). To date, no intrinsic candidates for limiting the hypertrophic response have been put forward.

S100 protein is a 20-kDa Ca2+-binding protein dimer composed of two subunits S100α and S100β, that have different tissue distributions (22–25). While human myocardium normally expresses S100α (24, 26), we detected, unexpectedly, in post-mortem myocardium of human subjects deceased with chronic lung disease, the presence of S100β, a subunit usually expressed in brain, presumably induced in heart prior to death by elevated levels of endogenous or exogenously administered catecholamines (27). S100β has been shown to bind specifically in vitro to several substrates of PKC inhibiting their phosphorylation by the enzyme, with a preferential inhibition of phosphorylation of some substrates by recombinant β-PKC relative to the α- and γ-PKC subtypes (28–31).

In the present article, report that, first, as in response to pressure overload, the induction of skACT in rat myocardium post-infarction is transient, and its down-regulation is temporally associated with the delayed induction of S100β. Second, transfection of an S100β expression vector into cultured neonatal rat cardiac myocytes inhibits the β-PKC mediated induction of β-MHC and skACT by NE and PE, respectively. Third,
hypoxia induces β-MHC transcription in cultured myocytes, a response similarly blocked by forced expression of S100β. These results suggest that the appropriately timed induction of S100β is part of an intrinsic negative regulatory pathway that limits the hypertrophic response following myocardial infarction.

MATERIALS AND METHODS

Rat Model of Myocardial Infarction—The experimental model of lateral wall myocardial infarction by surgical left coronary artery ligation was established in male Sprague-Dawley rats aged 12–14 weeks as described previously (1).

DNA Plasmids—A DNA construct DO3.44 encoding the complete amino acid sequence of human S100β flanked by 16 and 650 nucleotides of 5′- and 3′-untranslated sequences, respectively, was assembled by joining the cDNA clone KN3 (32) with the genomic EcoRI-BamHI fragment DO2 that is contiguous with the 3′-end of KN3 cDNA at the EcoRI site and extends to the BamHI site near the 3′-end of exon 3 of S100β (33) (see Fig. 2). A control DNA construct DO3.5 was assembled by joining DO2 with KN3 in the reverse orientation. The two constructs were inserted into the pECE expression vector (34) to derive plasmids pPDO3.44 (directing the expression of S100β) were inserted into the pECE expression vector (34) to derive plasmids pPDO3.44 (directing the expression of S100β) were inserted into the pECE expression vector (34) to derive plasmids pPDO3.44 (directing the expression of S100β), pPDO3.44 (directing the expression of S100β), and pPDO3.5, 2 pmol (9 μg) S100β mRNA, was measured using RNase protection assays to determine steady-state levels of S100β mRNA, S100β mRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were performed by modifying published conditions (38). Antisense riboprobes for the rat S100β (39), skACT (a gift from Dr. L. K. Karns, Laboratory of Molecular Neuro-Oncology, University of Virginia Health Sciences, Charlottesville, VA), and GAPDH (Ambion, Austin, TX) were labeled with [α-32P]UTP (800 Ci/ mmol, Amersham, Oakville, Ontario) by in vitro transcription with SP6 RNA polymerase of appropriate RNA synthesis vectors. The S100β, skACT, and GAPDH probes (2 ng, 106 dpm) were hybridized with 25 μg (S100β) or 15 μg (skACT, GAPDH) of total RNA from rat heart or brain, or rat tRNA for 18 h at 45 °C and RNase-resistant hybrids were recovered using a commercial kit (Ambion), analyzed on 8% urea, 6% polyacrylamide sequencing gels and visualized by autoradiography.

Detection of Human S100β mRNA by RT-PCR—RNA was isolated from fetal and neonatal rat hearts, cultured neonatal myocytes, as well as from hearts of experimental adult rats and sham-operated controls or from rat brain, and rat tRNA.

RESULTS

Induction of skACT and S100β in a Rat Model of Experimental Myocardial Infarction—The steady-state levels of skACT and S100β mRNAs were measured in a rat model of myocardial infarction resulting from coronary artery ligation (1). The skACT mRNA, normally present in fetal and neonatal, but absent from adult rat myocardium, was re-expressed up to 7 days post-infarction and absent at 21 and 35 days, suggesting a down-regulation of the mRNA (Fig. 1A). S100β mRNA, absent from normal heart, was induced beginning at 7 days and increased up to day 35 post-infarction (Fig. 1B). GAPDH steady state mRNA levels served as a control for the quality and loading of the mRNA.

Transfection of Cardiac Myocytes with an S100β Expression Vector—To investigate a possible functional role for S100β induction in cardiac myocytes surviving infarction, we took advantage of primary cultures of neonatal rat cardiac myocytes (8). As a first step toward investigating if S100β could modulate pathways associated with hypertrophy in cell culture, we verified the expression of S100β mRNA in cardiac myocytes following transfection with the human S100β expression plasmid pPDO3.44 (Fig. 2). Since 3–5% of cultured cardiac myo-
cytes are transfected by exogenous DNA (18), we used RT-PCR to demonstrate that pPDO3.44 directed the expression of human S100β mRNA following transfection (Fig. 3).

Inhibition by S100β of a Pathway Induced by α1-Adrenergic Agonists in Cardiac Myocytes—The uptake of co-transfected plasmids by the same competent cells in cardiac myocyte cultures allows the measurement of the modulation of reporter constructs by co-transfected signaling molecules in the setting of low transfection efficiency (18, 19). The use of reporter constructs in which cardiac-specific promoters are fused to CAT and expression vectors specifying putative signaling molecules in this system offers an effective strategy for dissecting the pathways associated with hypertrophy. While signal transduction pathways activated following myocardial infarction are likely to be multifactorial, α1-adrenergic stimulation of cardiac myocyte cultures results in a hypertrophic phenotype involving the reproducible induction of β-MHC and skACT (9, 11, 12, 19, 21), as described above for surviving myocardium following coronary artery ligation. To investigate whether S100β could modulate this specific pathway, we co-transfected cultured myocytes with β-MHC-CAT and pPDO3.44 prior to NE treatment. The approximately 3-fold induction of β-MHC-CAT by NE was inhibited by co-transfection with pPDO3.44, but not by pPDO3.5 or pECE (Fig. 4), suggesting that expression of S100β was responsible for the abrogation of the NE response. S100β had no effect on basal transcription from the β-MHC promoter. In parallel experiments, we showed that co-transfection of pPDO3.44 also inhibited the induction of skACT-CAT by PE (Fig. 5).

 Interruption by S100β of Signal Transduction by PKC in Cardiac Myocytes—The induction of β-MHC and skACT by α1-adrenergic agonists in cultured cardiac myocytes can be reproduced experimentally by transfecting these cells with a constitutively active β-PKC (ΔPKCβ), suggesting the involvement of the PKC signaling pathway in this induction (18, 19). To establish that S100β blocked the NE or PE activation of the β-MHC or skACT promoters, respectively, by interrupting the PKC signaling pathway, we examined the effect of co-transfection of a constitutively active mutant of β-PKC (ΔPKCβ) and S100β on β-MHC and skACT transcription. Transfection with ΔPKCβ (but not wild type β-PKC) induced the β-MHC and skCAT promoters approximately 2.5-fold (Figs. 6 and 7), as described previously (18, 19). Co-transfection with S100β blocked the induction of β-MHC-CAT and skACT-CAT by ΔPKCβ when the cells were concomitantly treated with NE and PE, respectively, or by increasing the extracellular Ca2+ concentration from 2 to 3 mM (Figs. 6 and 7). Since these treatments promote release of Ca2+ from intracellular stores (40), or increase extracellular Ca2+ influx, and by themselves (i.e. in the absence of co-transfection with S100β) do not block the induction of β-MHC or skACT by ΔPKCβ (data not shown), our results suggest that S100β interruption of the PKC signaling pathway is Ca2+ dependent. In additional experiments, we demonstrated that expression of S100β in cardiac myocytes did not block the induction of the α-MHC promoter by thyroid hormone, a non-PKC mediated signaling pathway (data not shown). This result confirmed the specificity of action of S100β on the PKC signaling pathway in cardiac myocytes and eliminated the possibility of a generalized inhibition of trans-activation.

Induction of β-MHC Expression by Hypoxia in Cultured Cardiac Myocytes Is Similarly Inhibited by S100β—To examine the potential role of hypoxia as an alternate mechanism for regulating the cardiac phenotype post-infarction, we examined the effect of hypoxia on β-MHC transcription and modulation by S100β in cultured myocytes. Hypoxia induced the β-MHC promoter approximately 2.5-fold with β-MHC transcription returning to baseline following 24 h of re-exposure to normal oxygen tension (Fig. 8). S100β overexpression significantly attenuated the hypoxic induction of the β-MHC promoter to approximately 1.3-fold over baseline. Confirming no effect on basal transcription, S100β did not alter β-MHC promoter activity under either normoxic conditions or following hypoxia.
with subsequent re-oxygenation. Hypoxia did not result in cellular necrosis as myocyte numbers in culture remained unchanged during the time course of these experiments (data not shown).

**DISCUSSION**

Acute myocardial infarction remains the leading cause of death in the developed world. Among survivors, subsequent morphological and biochemical alterations in spared cardiac muscle can lead to congestive heart failure, contributing to additional morbidity and mortality (41). In a rat infarction model, residual cardiac myocytes undergo hypertrophy and initiate an associated genetic program that is comparable to the response of the heart to pressure overload (1–7). This entails the re-expression of genes normally restricted to the fetus, exemplified by the isoform switch from adult α-troponin C to embryonic β-MHC (1) and induction of skACT (Fig. 1A). While some features of this response can be interpreted as adaptive (e.g. the more efficient utilization of energy by β-MHC), other gene products may be maladaptive and provoke progressive myocardial dysfunction (42). Potential signals evoking the hypertrophic response are comprised of direct stretch or endocrine and paracrine trophic effectors, including circulating catecholamines, mediated by signaling pathways involving PKC (8–19). Intrinsic negative modulators that limit the hypertrophic response and its potential long term complications have not been previously identified.

The existence of such negative modulators is suggested by results of experiments in both the acute pressure overload and myocardial infarction models of cardiac hypertrophy in the rat. In the pressure-overload model (5, 6), there is a rapid increase in ventricular mass over the first 4–8 days following aortic constriction, a more moderate increase over days 8–15, and a continuing slow increase up to day 40. In parallel with the onset of cardiac hypertrophy, there is a rapid induction of both fetal markers, β-MHC and skACT, over the first 4–6 days, and an approximate 25–50% decrease in their mRNA levels over days 4–11, coincident with a moderation in cardiac hypertrophy. Thereafter, the time course of the induction of the two markers diverges, with the β-MHC mRNA level remaining constant during the continuing slow hypertrophy of cardiac muscle, and the skACT mRNA level decreasing with time to 25% of its original level at day 40. The down-regulation of the two markers over days 4–11 immediately precedes the deceleration in the rate of increase of myocardial mass over days 8–15. In the myocardial infarction model, qualitatively similar results are observed following coronary artery ligation: a rapid induction of β-MHC and skACT mRNAs over 2–7 days, the persistence of β-MHC mRNA at a constant level up to day 35 (1), and a rapid down-regulation of skACT mRNA to undetectable levels at day 21 (Fig. 1A).

These results could be interpreted to indicate the waning of the hypertrophic response because of either normalization of wall stress, or the withdrawal of trophic effectors. However, two observations in the post-infarct setting raise the possibility that countervailing negative regulatory mechanisms also play a role in modulating biochemical and morphological changes in myocardium. First, we have previously reported that exercise training instituted after experimental myocardial infarction limited ventricular dilatation and promoted a 30% decrease in β-MHC mRNA levels (1). Second, we have shown in the present article, that down-regulation of skACT mRNA in the acute post-infarction period, coincided with the induction of S100β, a protein normally expressed in brain (Fig. 1B).

To conclude that timely induction of S100β post-infarction could play a role in modulating the hypertrophic response, it was necessary to provide a biochemical link between S100β and
FIG. 6. Induction of the β-MHC promoter by a constitutively active β-PKC (ΔPKCβ) is inhibited by co-transfection with an S100β expression vector in the presence of NE or an increased extracellular Ca²⁺ concentration. Duplicate cultures of cardiac myocytes were co-transfected with β-MHC-CAT and RSV-LUX together with expression plasmids SRe-PKCβ (specifying wild type β-PKC) or SRe-ΔPKCβ (specifying a constitutively active β-PKC), and, in addition, where shown, with pCE or pPD03.44. As indicated, in some experiments, cultures co-transfected with pPD03.44 were concomitantly treated with NE (20 μM), or exposed to a higher extracellular Ca²⁺ concentration (increased from 2 to 3 mM), for 48 h following transfection. Bars denote mean ± S.E. of the ratio of CAT activities of experimental to control cultures (co-transfected with the expression vector SRe, β-MHC-CAT, and RSV-LUX, and treated with vehicle) (set at 1.0) of four independent experiments. The significant differences between experimental and control cultures are indicated by * (p < 0.05).

FIG. 7. Induction of the skACT promoter by a constitutively active β-PKC (ΔPKCβ) is inhibited by co-transfection with an S100β expression vector in the presence of PEB or an increased extracellular Ca²⁺ concentration. Duplicate culture of cardiac myocytes were co-transfected with skACT-CAT and RSV-LUX together with expression plasmids SRe-PKCβ (specifying wild type β-PKC) or SRe-ΔPKCβ (specifying a constitutively active β-PKC) and, in addition, where shown, with pCE or pPD03.44. As indicated, in some experiments, cultures co-transfected with pPD03.44 were concomitantly treated with PEB (20 μM) or exposed to a higher extracellular Ca²⁺ concentration (increased from 2 to 3 mM) for 48 h following transfection. Bars denote mean ± S.E. of a ratio of CAT activities of experimental to control cultures (co-transfected with the expression vector SRe, skACT-CAT, and RSV-LUX, and treated with vehicle) (set at 1.0) of four independent experiments. The significant differences between experimental and control cultures are indicated by * (p < 0.05).

signaling pathways mediating the induction of β-MHC and skACT. Studies in cultured neonatal rat cardiac myocytes, confirmed in the present work, indicate that β-MHC and skACT are induced both by α₁-adrenergic agonists and activation of the β-PKC signaling pathway (9, 12, 18, 19). While activation of β-PKC by α₁-adrenergic agonists in the course of induction of β-MHC and skACT has not been formally demonstrated, a number of experimental observations strongly suggest that the transcription of β-MHC and skACT genes in response to these effectors is mediated by activation of the β-PKC signaling pathway. First, the presence of β-PKC and its translocation between cellular compartments after treatment with NE have been demonstrated in cultured myocytes (43). Second, both α₁-adrenergic agonists and activated β-PKC stimulate transcription from the β-MHC and skACT promoters through a common response element (M-CAT) that binds the transcriptional enhancer factor-1 (18–21). In addition, a mutation in M-CAT that disrupted transcriptional enhancer factor-1 binding abolished both the α₁-adrenergic and β-PKC induction of the β-MHC promoter (21). In the present study, we show that co-transfection with an S100β expression vector inhibits induction of the β-MHC and skACT promoters by both α₁-adrenergic agonists and activated β-PKC (Figs. 4–7). This supports the notion that the α₁-adrenergic induction of these genes in cardiac myocytes is mediated by activation of β-PKC signal transduction pathways and provides a biochemical link to substantiate a role for S100β in negative regulation of the cardiac hypertrophic response following myocardial infarction.

Elevation of circulating catecholamines following myocardial infarction in vivo may contribute to hypertrophy in surviving myocytes as selective α₁-adrenergic blockade limits development of left ventricular hypertrophy in rats following coronary artery ligation (44, 45). However, surviving myocytes are subject to other stimuli which may regulate phenotype including, among others, relative hypoxia or ischemia in the peri-infarct zone. Mild hypoxia results in hypertrophy of cultured neonatal myocytes and induction of skeletal α-actin (46). Indeed, we provide the first data in cultured myocytes that hypoxia alone reversibly activates β-MHC transcription as further mechanistic insight into in vivo observations of gene expression changes post-infarction. That S100β inhibition of β-MHC transcription is not limited to induction by α₁-adrenergic agonists but also encompasses modulation of regulated gene expression by hypoxia provides further evidence that this protein may function as an inhibitor of the hypertrophic phenotype in vivo where trophic signaling is complex and multifactorial. Of interest, hypoxia results in translocation of PKC isoforms from the soluble to the particulate fraction in cultured neonatal cardiac myocytes and thus regulation of PKC may be a conserved mechanism for the effects of S100β on cardiac gene expression (47).

In cultured cardiac myocytes, it can be shown that S100β inhibits the induction of β-MHC and skACT in a similar fashion (Figs. 4–7). In vivo, after an initial rapid coordinate induction in response to myocardial infarction, the subsequent time
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The activities of an array of positive effectors, distinct from rapidly (Fig. 1). This divergence likely reflects the combined sumed increases in intracellular Ca\(^{2+}\) and inhibits their phosphorylation by the enzyme (28–31), it is indicated by co-transfection with an S100 1.5% CO\(_2\), 98.5% air) conditions alone or 18 h of hypoxia followed by courses of hypoxia or adrenergic stimulation or hypoxia, and negative regulators, that are unleashed following myocardial infarction and modu-lation of S100b-MHC remains constant (1) and that of skACT decreases in the post-mortem hearts of human subjects deceased with chronic lung disease (27), a reasonable postulate is that the induction of S100β is mediated by circulating catecholamines that are released following myocardial infarction (44). It is not known where S100β intersects the β-PKC signaling pathway. Since in vitro S100β binds to specific PKC substrates and inhibits their phosphorylation by the enzyme (28–31), it is tempting to speculate that S100β also interacts with an intracellular PKC substrate in cardiac myocytes, possibly a key intermediate transcription factor such as transcriptional enhancer factor-1 (19). This possibility can be tested experimentally in co-transfection experiments with transcriptional enhancer factor-1 expression vectors. It is interesting that the inhibition by S100β of the induction of β-MHC and skACT by the constitutively active β-PKC (ΔPKCβ) required the concomitant treatment with α\(_1\)-adrenergic agonists or an increase in the intracellular Ca\(^{2+}\) concentration in the medium from 2 to 3 mM (Figs. 6 and 7). Since the myocytes remained viable and beating during these treatments, we estimate, based on published data (48), that they could have tolerated transient increases in the intracellular Ca\(^{2+}\) concentration from a basal level of 0.1 to 0.2–10 μM in response to treatment with α\(_1\)-adrenergic agonists (40) or an increase in the extracellular Ca\(^{2+}\) concentration. These presumed increases in intracellular Ca\(^{2+}\) levels are well within the range required to stimulate the interaction of S100β with other proteins in vitro in carefully titrated reactions (30, 49, 50). In the context of the hypertrophic response in post-infarcted cardiac muscle in vivo, the inhibition of the PKC signaling pathway by S100β may depend on parallel alterations in Ca\(^{2+}\) handling, including augmented resting Ca\(^{2+}\) concentration previously demonstrated in ischemic heart failure (51).

While several positive effectors and signal transduction pathways that initiate and sustain the myocardial hypertrophic response have been identified (15), S100β is the first candidate for an intrinsic negative regulator of this response through inhibition of the PKC signaling pathway. This opens up the way for the search and identification of other negative regulators of the myocardial hypertrophic response to acute ischemic injury.

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