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Epinephrine-induced MMP expression is different between skeletal fibroblasts and myoblasts

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Skeletal fibroblasts and myoblasts are among the cell types currently being considered in cell therapy for ischaemic heart disease. To investigate whether the expression of the tissue-remodelling proteolytic enzymes matrix metalloproteinases (MMPs) and the cellular energy regulator AMP-activated protein kinase (AMPK) is comparable between the two cell lines in response to epinephrine treatment, mouse skeletal fibroblasts (NOR-10) and myoblasts (C2C12) were treated with or without a low (11 nmol·l^{-1}) or high (55 nmol·l^{-1}) dose of epinephrine for 2 or 6 h. Cellular MMP-3 expression was increased by the high-dose epinephrine at both treatment periods in both cell lines. Cellular MMP-2 and MMP-13 expressions were amplified by the 2- or 6-h epinephrine incubation in fibroblasts. However, in myoblasts, such an increase was only seen at the longer treatment time. An elevated AMPKα expression was observed after a 2-h presence of epinephrine in both cell lines, which matches temporally with the early increased cellular MMP-2 and MMP-13 expression in fibroblasts. Activity of secreted MMP-2 increased only after 6-h epinephrine treatment in both cell types. Our data suggest that skeletal fibroblasts respond earlier to epinephrine application in terms of endogenous synthesis of the proteolytic and the energy homeostasis enzymes, whereas such response occurs later and to a milder dose of the beta adrenergic agonist in myoblasts. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—matrix metalloproteinase; Adenosine monophosphate (AMP)-activated protein kinase; epinephrine; myoblast; fibroblast; zymography

INTRODUCTION

In the last decade, cell therapy has emerged as a promising approach for both prevention and treatment of ischaemic heart disease.[1,2] Several cell lines including skeletal myoblasts and fibroblasts have been tested for such therapeutic potentials by both *in vitro* or clinical trials. The initial premise is that the transplanted exogenous cells can engraft and integrate with host cardiomyocytes for cardiac regeneration. Later experimental studies suggest multiple mechanisms including enhancement of neovascularization and remodelling of extracellular matrix by inducing expression and regulation of matrix metalloproteinases (MMPs).[3,4]

Matrix metalloproteinases are zinc-dependent endopeptidases, which are capable of degrading extracellular matrix components. MMPs are synthesized as inactive zymogens, which require activation by extracellular proteases. MMP activities are tightly regulated by endogenous tissue inhibitors of metalloproteinase.[5] A large body of experimental evidence has confirmed the roles of MMPs in physiological tissue development and growth, wound healing, extracellular matrix turnover and homeostasis. MMPs are required in the process of myogenesis during embryonic development and muscle hypertrophy and hyperplasia during exercise.[6] MMPs also play a crucial role in angiogenesis in cardiac muscle remodelling following myocardial infarction.[7,8]

AMP-activated protein kinase (AMPK) is a master regulator of cellular energy homeostasis. AMPK is activated by an increase in intracellular AMP concentration because of hormonal stimulus (e.g. epinephrine) or stresses, such as hypoglycemia, strenuous exercise or ischaemia. Upon activation, AMPK enhances glucose and fatty acid uptake and ATP-producing catabolic pathways such as fatty acid oxidation and ketogenesis, while suppressing ATP-consuming anabolic pathways including lipogenesis, protein and fatty acid synthesis. Although the physiological role played by this enzyme during health and disease is far from being clearly defined, AMPK is suggested to contribute to cell survival and increase heart tolerance to ischaemia.[9,10] As reviewed by Musi *et al*. (2003)[11] and Jorgenson *et al*. (2006),[12] accumulating evidence additionally suggests that activation of AMPK has implications on the expression of a large number of genes. The AMPK catalytic subunit ARK5 has been shown to induce activation of MMP-2 and MMP-9.[13] Therefore, AMPK protein also may play a role in signalling MMPs' expression.

Epinephrine is an acute stress response hormone secreted from the adrenal gland. Epinephrine synthesis is under the control of hypothalamus adrenocorticotropic hormone (ACTH) axis and sympathetic nervous system and is

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responsive to varied physiological challenges such as exercise, noise, extreme environment temperature and psychological stress. In addition, it has been shown that circulating epinephrine levels are increased in some common life-threatening diseases such as heart attack, chronic heart failure and cancer.[14–17] The current study was designed to examine whether the expression of MMPs, the extracellular matrix remodelling enzymes, and the cellular energy regulator AMPK is different between fibroblasts and myoblasts in response to epinephrine at concentrations observed during physiological stress or after myocardium infarction. The relationship between the expression of MMPs and AMPK in response to the elevated adrenal hormone also was investigated.

MATERIALS AND METHODS

Cell lines and reagents

Mouse skeletal fibroblasts (NOR-10) and myoblasts (C2C12) were purchased from the American Type of Cell Culture (ATCC, Manassas, VA, USA). The NOR-10 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 20% fetal bovine serum, and C2C12 cells were cultured in DMEM with 10% fetal bovine serum. The anti-AMPKα rabbit monoclonal antibody (23A3) and anti-rabbit immunoglobulin G horse radish peroxidase (HRP)-linked secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Fluorescein conjugate DQ gelatin was purchased from Molecular Probes Inc (Eugene, OR, USA).

Epinephrine treatment

Equal numbers (8×10^5) of cells were seeded into 60-mm culture dishes and grew over night. For the excreted MMP assays, cells were then washed twice with phosphate-buffered saline (PBS) and cultured in serum-free medium. For cellular MMPs and AMPKα assays, the overnight culture medium was replaced by fresh medium containing serum. Epinephrine (final concentration of 11 or 55 nmol·l^{-1}, Sigma) was added to the culture medium and the cells were cultured for additional 2 or 6 h. The doses chosen for epinephrine application are based on the report that serum epinephrine concentration increased from 0·5 nmol·l^{-1} at resting condition to 10 nmol·l^{-1} or higher in intensive exercise and over 50 nmol·l^{-1} immediately after a myocardial infarction.[18] Our preliminary experiments demonstrated that epinephrine at those doses lost its potency at 12-h or longer incubation time on cellular expression of AMPKα in both cell lines. Therefore, we chose 2 and 6 h as the treatment period. Control cells were cultured by the same way but without adding epinephrine. The serum-free culture medium in the secreted MMP assay was collected and stored at −80 °C for further assay. Cell pellets were lysed on ice by sonification in a cold PBS buffer with added 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS) and 0·5% deoxycholic acid. The protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad).

Zymography assay

The expression of MMPs in the culture medium and cell lysates were determined using gelatin (or casein for MMP-3) zymography carried out with minor modification of the method described by Seftor and coworkers.[19] Briefly, 0·1% of gelatin was co-polymerized in 10% polyacrylamide gel. Cell culture medium (volumes normalized for equal number of cells/well) or cell lysate (30 μg protein/lane) was mixed with the non-reducing sample buffer (Bio-Rad, Hercules, CA, USA) and loaded into the gels and run at 100 V for about 2 h at 4 °C. In casein zymography, the gels were pre-run at 100 V for 20 min. The gel was then washed with 2·5% Triton-X-100 for 0·5 h and incubated with zymography incubation buffer (Bio-Rad) at 37 °C for 18–24 h. The gels then were stained with 0·5% Coomassie blue solution for 0·5 h and destained by a solution containing 10% acetic acid and 40% methanol. The gels were scanned, and the bands in the digital image were analysed using Image J software (NIH Image, National Institutes of Health).

Western blot assay

Cells lysate was loaded into 10% acrylamide gels, and proteins were separated by SDS-polyacrylamide gel electrophoresis at 150 V for 1·5 h. The proteins were then transferred onto polyvinylidene fluoride (PVDF) membrane and blocked with 5% milk in Tris-buffered saline containing 0·05% Tween-20 (TBS-T) for 1 h. The membrane was then probed with the AMPKα monoclonal antibody overnight at 4 °C with gentle shaking. The antibody detects both the α1 and α2 isoforms of the catalytic subunit. On the second day, membranes were washed with TBS-T for 0·5 h and incubated in the secondary antibody conjugated to horseradish peroxidase for 1 h. Blots were developed using the enhanced chemiluminescence method (Amersham, Buckinghamshire, UK) and exposed to film in a dark room. The signal density in the bands was analysed on a digitised image of the film using Image J.

Cell in situ zymography assay

Cell *in situ* zymography was performed following a method developed in our laboratory.[20] Briefly, cells were seeded onto glass cover slips and grew overnight in culture dishes. Cells were then treated with or without 55 nmol·l^{-1} epinephrine for 6 h. The cells were fixed with 4% paraformaldehyde and embedded into a mixture of 0·5% of agarose and 10% fluorescein conjugate DQ at 37 °C overnight. The gelatinolytic activity was examined using a fluorescence microscope equipped with an emission filter of 515 nm and an absorption filter of 495 nm, and images captured using a digital camera attached to the microscope.

Cell lysate gelatinolytic activity assay

Cell lysates of epinephrine-treated or non-treated control cells was used to determine the total gelatinolytic activity using the EnzChek gelatinase/collagenase assay kit. The fluorescence intensity was determined using a Synergy HT fluorescence microplate reader (BioTek, VT, USA).

Data analysis

Data were analysed separately according to individual cell lines and treatment length using one-way analysis of variance (epinephrine dose as the main effect) using an SPSS general linear model program (SPSS Inc., Chicago, IL, USA). For AMPKα, the data were analysed separately according to individual cell lines and epinephrine dose using the length of treatment as the main effect. Means were separated using Fisher's protected least significant difference test.

RESULTS

Intracellular MMPs are the larger, inactive pro-forms of the enzyme. Bands shown on zymograms from cell lysates are caused by the activation of the proenzyme by SDS in the cell lysate buffer and electrophoresis running buffer. The caseinolytic expression of intracellular MMP-3 was increased by the presence of high-dose (55 nmol·l^{-1}) epinephrine at both lengths of treatment in both cell lines. A lower dose of epinephrine (11 nmol·l^{-1}) and longer time of treatment (6 h) also enhanced the protease expression in myoblasts (Figure 1).

Figures 2 and 3 show the cellular expressions of MMP-2 and MMP-13 in fibroblasts and myoblasts. On the gel image, bands on the top row are the MMP-2 (64 kDa), and bands on the bottom are the MMP-13. At the higher dose, the expression of the two enzymes was elevated at both lengths of epinephrine treatment in fibroblasts, but in myoblasts, this is only seen in the longer time (6 h) treatment.

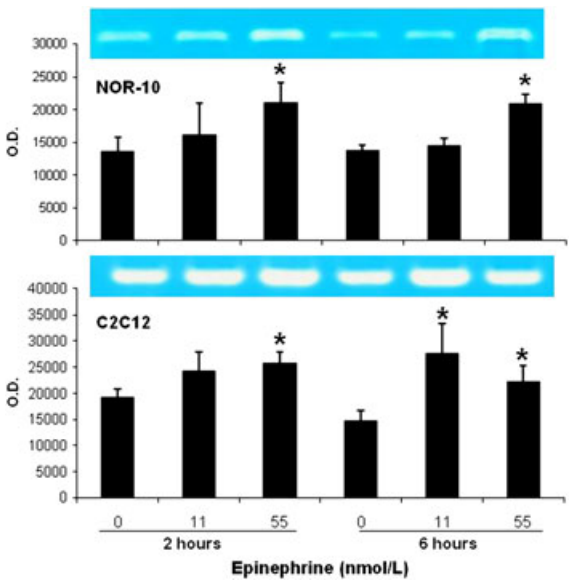

Figure 1. Casein zymography shows the cellular expression of matrix metalloproteinase (MMP) 3 in mouse skeletal fibroblasts (NOR-10) and myoblasts (C2C12) in response to epinephrine treatment. Cell culture media were supplemented with or without 11 or 55 nmol·l^{-1} of epinephrine and cultured for additional 2 or 6 h. Data presented are means from a minimum of three independent experiments. *Significantly different from the non-treated control in the same length of time treatment [$p < 0{\cdot}05$, Fisher's protected least significant difference (LSD) test]

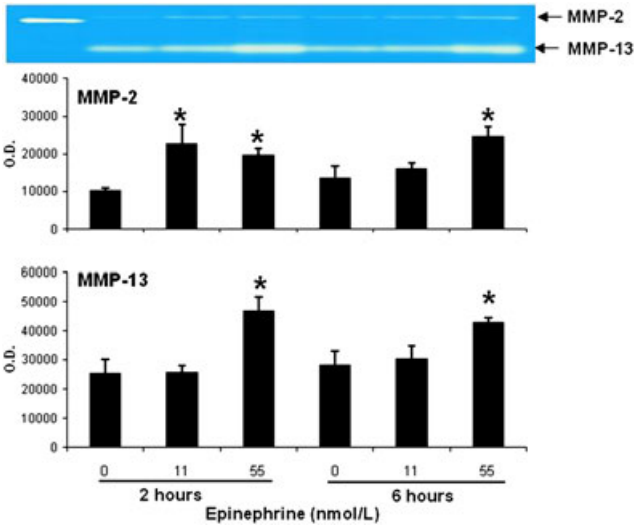

Figure 2. Gelatin zymography shows the cellular expression of MMP-2 and MMP-13 in NOR-10 fibroblasts in response to epinephrine treatment. Cell culture media were supplemented with or without 11 or 55 nmol·l^{-1} of epinephrine and cultured for additional 2 or 6 h. Data presented are means from a minimum of three independent experiments. The band shown on the top left of the gel image is the active human recombinant MMP-2 (66 kDa). *Significantly different from the non-treated control in the same length of time treatment within same cell line ($p < 0{\cdot}05$, Fisher's protected LSD test)

At the lower dose, MMP-2 and MMP13 expressions were increased by 6 h of incubation with epinephrine in myoblasts but not in fibroblasts.

The activity of the secreted MMP-2 in the culture medium was increased only after 6 h of epinephrine treatment in both

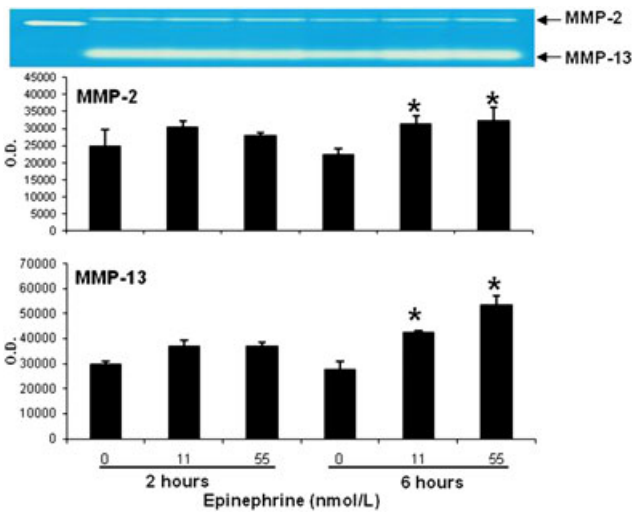

Figure 3. Gelatin zymography shows the icellular expression of MMP-2 and MMP-13 in C2C12 myoblasts in response to epinephrine treatment. Cell culture media were supplemented with or without 11 or 55 nmol·l^{-1} epinephrine and cultured for additional 2 or 6 h. Data presented are means from a minimum of three independent experiments. The band shown on the top left of the gel image is the active human recombinant MMP-2 (66 kDa). *Significantly different from the non-treated control in the same length of time treatment within the same cell line ($p < 0{\cdot}05$, Fisher's protected LSD test)

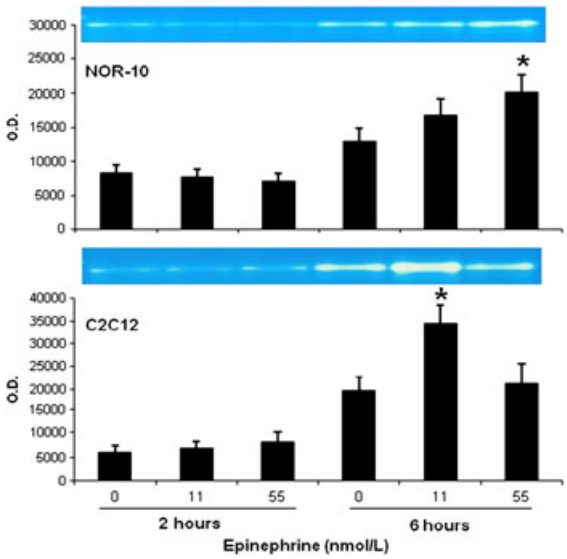

Figure 4. Gelatin zymography shows the activity of secreted MMP-2 from NOR-10 fibroblasts and C2C12 myoblasts in the culture medium. Cells were seeded in culture dishes for overnight. After being washed with phosphate-buffered saline, the cells were cultured in serum-free conditioned medium supplemented with or without 11 or 55 nmol·l^{-1} of epinephrine and cultured for additional 2 or 6 h. Data presented are means from a minimum of three independent experiments. *Significantly different from the non-treated control in the same length of time treatment within the same cell line ($p < 0{\cdot}05$, Fisher's protected LSD test)

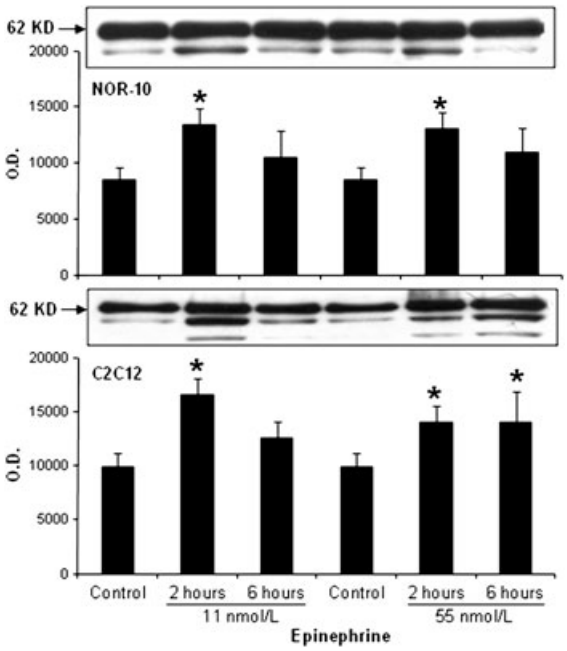

Figure 5. Western blots show the AMPKα expression in NOR-10 fibroblasts and C2C12 myoblasts in response to epinephrine treatment. Cell culture media were supplemented with or without 11 or 55 nmol·l^{-1} of epinephrine and cultured for additional 2 or 6 h. AMPKα and its post-translation modified (cleavage, splicing) proteins with lower molecular weights were recognized by the antibody as shown in the gel image. Data are the sum of the optical density from the signal bands of original AMPKα (62 kDa) and other smaller metabolic peptides. Data presented are means from a minimum of three independent experiments. *Significantly different from the non-treated control in the same dose group within same cell line ($p < 0{\cdot}05$, Fisher's protected LSD test)

cell lines (Figure 4). An elevated amount of MMP-2 was found in the medium of the higher dose-treated fibroblasts and the lower dose-treated myoblasts.

An early response of AMPKα expression in reaction to epinephrine application was observed in both fibroblasts and myoblasts. The amount of the enzyme was increased by both the lower and the higher dose of the hormone supplement (Figure 5). Epinephrine lost its potency in terms of inducing AMPKα expression after 6-h incubation of fibroblasts. In contrast, the expression of this enzyme remained higher as compared with the control after the longer time and higher dose epinephrine treatment in myoblasts.

Cell *in situ* zymography images are shown in Figure 6. There are highlighted fluorescent dots spreading at certain areas of cytoplasm in the epinephrine-treated cells of both cell lines, but this is not seen in the control cells. This implies that at first 6h, the newly synthesized gelatinase/collagenase in response to epinephrine treatment is highly concentrated and stays in the rough endoplasmic reticulum locally without transporting or excreting. The lower panel in this figure shows that the gelatinolytic activity in the lysate of cells treated with epinephrine is higher than that in the non-treated control in both cell lines.

DISCUSSION

Cell transplantation has been proposed as a therapeutic strategy for cardiac regeneration after myocardial infarction. Basic research and clinical trials have demonstrated promising results for both the prevention and treatment of heart failure. Earlier studies investigated structural changes in cardiomyocytes to explain the potential benefits for myocardial contraction. Recent research suggests that the beneficial effects are primarily dependent on the paracrine effect of transplanted cells on extracellular matrix remodelling and vascularization.[8] Although skeletal myoblasts have been the widely used cells in cell-based therapy for cardiac repair and regeneration, limited information is available regarding their energy homeostasis and paracrine activity of proteolytic enzymes in facing emergency situations. This current research demonstrates that as compared with skeletal fibroblasts (another type of cells closely localized with muscle cells in the muscle), a longer period of increased AMPKα expression and a delayed response of intracellular synthesis of MMP-2, MMP-3 and MMP-13 was observed in myoblasts in response to a high physiological concentration of epinephrine. In considering AMPK's contribution to caloric intake and energy balance as well as the paracrine actions of MMPs on the extracellular matrix remodelling and angiogenesis, it seems that skeletal myoblasts perform better than fibroblasts in terms of cell survival and myocardium regeneration.

Remodelling of connective tissue extracellular matrix is a fundamental requirement for many physiological and pathological processes such as muscle development and

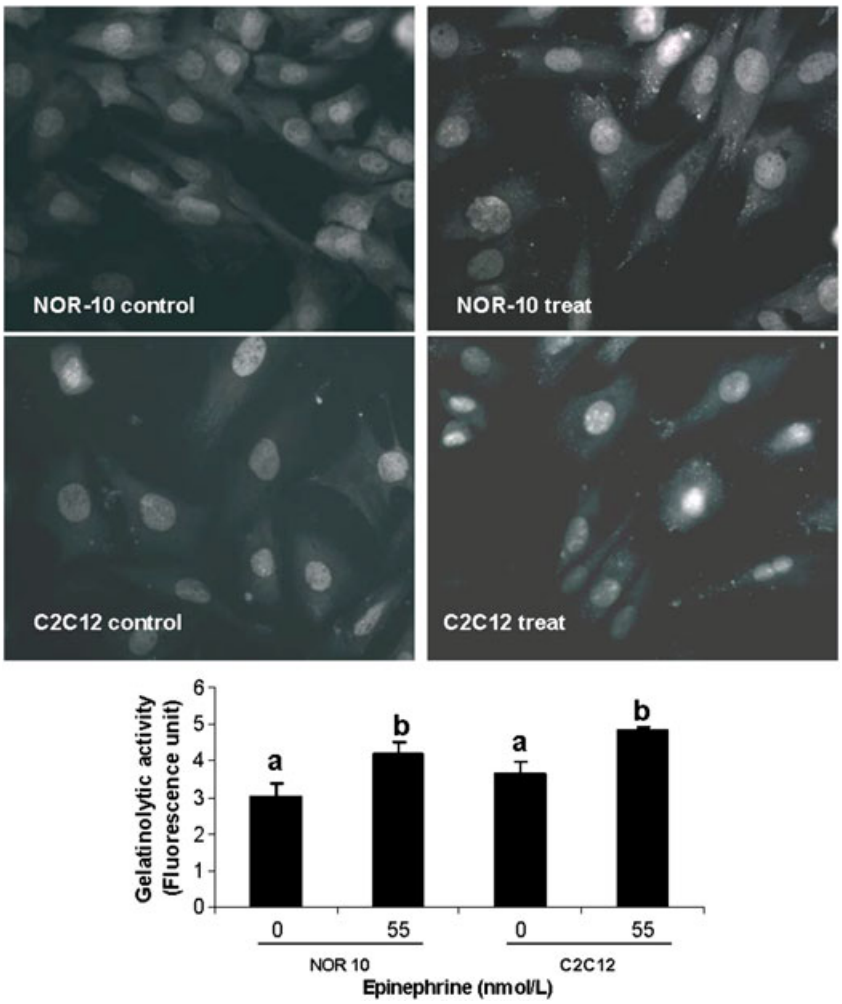

Figure 6. Cell *in situ* zymography shows the gelatinolytic activity by NOR-10 fibroblasts and C2C12 myoblasts in response to epinephrine treatment. Cells seeded on top of glass coverslips were cultured for 6h in medium supplemented with (treated) or without (control) 55 nmol·l^{-1} of epinephrine. The cells were then embedded into a mixture of agarose and fluorescein conjugate DQ gelatin and incubated at 37°C for overnight. The gelatinolytic activity was examined using a fluorescence microscope. The bottom panel shows the total gelatinolytic activity of lysates of cells treated with or without 55 nmol·l^{-1} of epinephrine for 6h. Data presented are means from three independent experiments. Means labelled with different letters within the same cell line are significantly different ($p < 0{\cdot}05$, Fisher's protected LSD test)

growth, post infarct myocardium remodelling, atherosclerotic plaque formation and cancer cell invasion. This process requires the activity of various proteolytic enzyme families, of which the MMPs play a critical role. The expression of MMPs is regulated by many biological factors including the adrenal gland-originating epinephrine, the circulating concentration of which is controlled by physiological and pathological conditions. Previous studies demonstrate that exercise, as well as myocardiac infarction, increased epinephrine secretion, and elevated epinephrine all in turn boosted MMP expression.[15,16,21] The current research further demonstrates that skeletal fibroblasts and myoblasts show differences in cellular MMP expression and extracellular secreted MMP's activity in response to epinephrine treatment *in vitro*, and such differences are interactively determined by the length of treatment and the concentration of the hormone.

It has been shown that overexpression of osteoactivin, a type I membrane glycoprotein, induced expression of MMP-3 in NIH-3T3 fibroblasts but not in C2C12 myoblasts.[22] Again, cAMP activated the activity of ERK1/2, the extracellular signal-related kinase, in fibroblasts but not in myoblasts.[23] We have previously shown that MMP-9 activity in fibroblasts and myoblasts reacts differently to substrate coating and mechanical stretching.[24] The current results demonstrate that, at the shorter incubation time, epinephrine induced increases in expression of intracellular MMP-2, the most prominent MMP in terms of cell migration, in fibroblasts. However, under this treatment regime, the activity of secreted MMP-2 is not increased in the culture medium, suggesting there is a time delay from endogenous synthesis to exocytosis of this proteolytic enzyme, and this process also is determined by cell phenotype. A longer time incubation with the lower dose epinephrine led to an increase in cellular

MMP-2 expression in myoblasts but not in fibroblasts. After 6 h of epinephrine treatment, the overexpressed intracellular MMP-2 in myoblasts is secreted into the medium to perform its biological function. This finding suggests that, in response to a concentration of epinephrine stimulated by stress, myoblasts but not fibroblasts are responsible for the extracellular MMP-2 activity.

Two other MMPs expressed abundantly in our cell lines are MMP-13 and MMP-3. MMP-13 is a collagenase that plays a primary role in extracellular matrix and tissue remodelling in some pathological processes such as cancer and arthritis.[25] MMP-3, also called stromelysin-1, plays a role in initiation of cell migration. In our experiment, the lower-dose epinephrine did not enhance the expression of MMP-13 in fibroblasts but boosted its appearance at a longer treatment time in myoblasts. Similar findings also are observed for MMP-3 expression. This result again suggests that myoblasts, not fibroblasts, are responsible for the extended response of MMP expression in reaction to an application of a high physiological dose of epinephrine.

AMP-activated protein kinase is a key enzyme controlling cellular energy homeostasis and a master regulator of cellular metabolism during stress response. It has been demonstrated that activation of AMPK significantly improved left ventricular function and survival in experimental heart failure mice[26] and delayed apoptosis under anoxic conditions in cardiac cells.[27] In addition, increased expression of functional AMPK helped resistance to apoptosis in skeletal myoblasts in facing serum starvation.[28] In the current experiment, the elevated expression of AMPKα was longer lasting in myoblasts than in fibroblasts in response to the emergency hormone. We can reasonably speculate that myoblasts can better survive in facing transplantation stress than fibroblasts because of a better regulation of their cellular energy metabolism. In addition to energy metabolism, AMPK may play a role in MMP expression. It has been shown that increased expression of ARK5, another AMPK catalytic subunit, induced MMP-2 and MMP-9 synthesis in human pancreatic cancer cells.[13] The reported ARK5 signalling on MMP expression provides a connection between the cellular energy metabolism and tissue remodelling. In this study, the better correlation between the expression of MMPs and AMPK catalytic subunit AMPKα in fibroblasts as compared with that in myoblasts suggests that such AMPK signalling in MMP synthesis may be determined by cell type.

In summary, although skeletal muscle fibroblasts and myoblasts are closely localized *in vivo*, and both potential cell transplantation candidates for cardiac regeneration after infarction, expression of the extracellular proteases as well as the cellular energy metabolism enzymes is different in response to the adrenal beta-agonist epinephrine. Increased expressions of MMP-2 and MMP-13 tend to occur earlier in the fibroblasts, corresponding well with the epinephrine-induced expression of cellular AMPK. In contrast, a delayed increase of both intracellular and secreted MMP expressions in response to a high physiological concentration of epinephrine and a sustained overexpression of AMPK are demonstrated in myoblasts. This result suggests that, as compared with fibroblasts, skeletal myoblasts can perform better in terms of proteolytic paracrine action and cellular energy metabolism when transplanted into infarcted myocardium.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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