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Regulation of glucose transporter expression in cardiac myocytes: p38 MAPK is a strong inducer of GLUT4

Christophe Montessuit*, Nathalie Rosenblatt-Velin[1], Irène Papageorgiou, Lis Campos, Corinne Pellieux, Tatiana Palma, René Lerch

Division of Cardiology, Department of Internal Medicine, Geneva University Hospitals, Rue Micheli-Du-Crest 24, 1211 Geneva 14, Switzerland

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Abstract

Objective: In vivo differentiation of cardiac myocytes is associated with downregulation of the glucose transporter isoform GLUT1 and upregulation of the isoform GLUT4. Adult rat cardiomyocytes in primary culture undergo spontaneous dedifferentiation, followed by spreading and partial redifferentiation, which can be influenced by growth factors. We used this model to study the signaling mechanisms modifying the expression of GLUT4 in cardiac myocytes. **Results:** Adult rat cardiomyocytes in primary culture exhibited spontaneous upregulation of GLUT1 and downregulation of GLUT4, suggesting resumption of a fetal program of GLUT gene expression. Treatment with IGF-1 and, to a minor extent, FGF-2 resulted in restored expression of GLUT4 protein and mRNA. Activation of p38 MAPK mediated the increased expression of GLUT4 in response to IGF-1. Transient transfection experiments in neonatal cardiac myocytes confirmed that p38 MAPK could activate the *glut4* promoter. Electrophoretic mobility shift assay in adult rat cardiomyocytes and transient transfection experiments in neonatal cardiac myocytes indicated that MEF2 was the main transcription factor transducing the effect of p38 MAPK activation on the *glut4* promoter. **Conclusion:** Spontaneous dedifferentiation of adult rat cardiomyocytes in vitro is associated with downregulation of GLUT4, which can be reversed by treatment with IGF-1. The effect of IGF-1 is mediated by the p38 MAPK/MEF2 axis, which is a strong inducer of GLUT4 expression.
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Keywords: Gene expression; Growth factors; Cell culture/isolation; Cell differentiation; MAP kinase

1. Introduction

Cardiac myocytes use a variety of substrate for energy production, including free fatty acids, glucose, lactate and ketone bodies. Substrate selection is developmentally regulated. During the perinatal period, substrate metabolism shifts from predominant non-oxidative glucose utilization to predominant fatty acids oxidation [1]. This shift is associated with a change in the expression of a number of regulatory proteins of glucose and fatty acids metabolism [2–5], including glucose-transporting proteins. Specifically, the ubiquitous glucose transporter GLUT1 is largely replaced by the insulin-regulated isoform GLUT4 [2,3,5].

Myocardial hypertrophy is associated with changes in glucose metabolism characterized by increased basal glucose uptake and insulin resistance [6,7]. Basal uptake of glucose is mediated GLUT1, which is expressed at a low level in the normal adult heart, but is increased in rat models in vivo during post-ischemic reperfusion [8] or cardiac failure following remodeling after large myocardial infarction [9]. Conversely expression of GLUT4 is decreased [8,9]. Observations in GLUT4-null mice indirectly suggests that downregulation of GLUT4 may be involved in the pathogenesis of hypertrophy [10,11]. In addition, GLUT4 deficiency is associated with impaired post-ischemic recovery [12].

Adult rat cardiomyocytes (ARC) in primary culture re-express markers of hypertrophy, including ANF and α-

* Corresponding author. Tel.: +41-22-37-27-216; fax: +41-22-37-27-229.
E-mail address: christophe.montessuit@hcuge.ch (C. Montessuit).
[1] Present address: Division of Hypertension, University of Lausanne Medical School, Rue Du Bugnon, CH-1011 Lausanne, Switzerland.

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smooth muscle actin, which are normally expressed during fetal and perinatal life, but are downregulated during maturation [13]. The growth factors FGF-2 and IGF-1 modulate this process in opposite directions [14–16]. FGF-2 accelerates expression of markers of dedifferentiation, while IGF-1 promotes a more differentiated phenotype.

In the present study, we used the model of ARC in primary culture, first, to determine the influence of growth factors affecting differentiation of cardiac myocytes on expression of GLUT4 and, second, to decipher the signaling pathways involved in the control of expression of GLUT4.

2. Materials and methods

2.1. Animals

We obtained male Sprague–Dawley rats (100–110 g) from IFFA CREDO (L'Arbresle, France). Newborn Sprague–Dawley rats (1–3 days) were from the Geneva University School of Medicine animal facility. The ethical committee of the Geneva University School of Medicine and the Geneva State Veterinary Office approved the study protocol, which conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Adult rat cardiomyocytes culture

Adult rat cardiomyocytes (ARC) were isolated by retrograde perfusion of the hearts with collagenase (type II, Biochrom) [17,18]. Cells were plated onto dishes or chamber slides (Lab-Tek, Nalge Nunc) previously coated with 0.1% gelatin and incubated overnight with culture medium containing 20% fetal calf serum (FCS). Plating density was approximately 20,000 cells/cm^2. Culture medium was M199 with Earle's salts (Life Technologies) supplemented with 20 mM creatine, 100 μM cytosine-β-D-arabinofuranoside and 1% FCS.

Recombinant human Fibroblast Growth Factor-2 (FGF-2, Boehringer-Mannheim) and recombinant human Insulin-like Growth Factor-1 (IGF-1, Bachem, Basel, Switzerland) was added from 10 mM CH_3COOH stock solutions to the medium at the time of plating (day 0). The medium was replaced on day 2.

The MAP kinase inhibitors U0126 (Promega) and SB203580 (Calbiochem) were also added at the time of plating from DMSO stock solutions. The final concentration of DMSO in culture medium (0.1%) had no effect on the expression of GLUT4.

2.3. Neonatal rat cardiomyocytes culture

Neonatal rat cardiomyocytes (NRC) were isolated by collagenase digestion and maintained in Dulbecco's modified Eagle's medium (DMEM)/medium 199 (4:1) supplemented with penicillin and streptomycin [19]. Cells were plated at a density of 2.5×10^5 cells per 3.5-cm dish or 5×10^5 cells per 6-cm dish.

2.4. Plasmids and NRC transfection

Marcelle Lavau kindly donated the plasmid p2.2Luc-2212GLUT4 containing 2.2 kb of the rat *glut4* promoter [20]. We excised the *glut4* promoter with *Kpn*I and *Nhe*I and inserted it into the multiple cloning site of pGL3basic (Promega). The resultant plasmid pLuc-GT4 encodes *Photinus* luciferase under the control of the rat *glut4* promoter. Using the QuickChange mutagenesis kit (Stratagene), we mutated the MEF2 responsive element in position −466 to −457 in the rat *glut4* promoter from CTAAAAATAG to CTAGCCTTAG to generate the plasmid pLuc-GT4-MEF2-mut. Andrew Thorburn kindly provided the pSRα-MEK6DD plasmid encoding a constitutively active mutant of MEK6 [21]. All plasmids were cotransfected with a plasmid encoding *Renilla* luciferase under the control of the SV40 promoter (pRL-SV40, Promega). Transient transfection of NRC was performed using the calcium phosphate precipitation method [19]. Transfection mixes contained 1 μg of pLuc-GT4, 1 μg of pRL-SV40 and 1 μg of the selected expression plasmid or the empty vector. *Photinus* and *Renilla* luciferase activity was measured 2 days after transfection with the Dual Luciferase Reporter kit (Promega).

2.5. Immunoblot analysis

ARC in one 6-cm dish were extracted in 200 μl RIPA (150 mM NaCl, 9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, pH 7.4, 1:100 protease inhibitor cocktail (Sigma P8340), 5 mM NaF, 10 mM Na_2β-glycerophosphate, 10 mM Na_2paranitrophenyl phosphate, 1 mM $NaVO_3$). Extracts were clarified by centrifugation (12,000 rpm for 5′) and supernatants were used for SDS-PAGE and immunoblotting [22]. Antibodies against phosphorylated and non-phosphorylated forms of ERK1/2, Akt and p38 MAPK were from Cell Signalling Technologies (Beverly, MA). A rabbit polyclonal antibody against GLUT1 was obtained from Diagnostic International (Schriesheim, Germany). A monoclonal anti-GLUT4 antibody (clone 1F8) was obtained from ANAWA (Wangen, Switzerland). Anti-rabbit and anti-mouse IgG HRP-conjugated antibodies were from Sigma. Blots were revealed by chemiluminescence (ECL, Amersham). Quantitative analysis of bands on films was done by laser densitometry (ImageQuant 3.3, Molecular Dynamics).

2.6. RT-PCR analysis

ARC or NRC in one 6-cm dish were extracted in 1 ml TRIZOL (Life Technologies) and total RNA was purified

according to the manufacturer's protocol. Two kinds of RT-PCR analysis were performed. In the first approach, 125 ng total RNA was used for reverse transcription and subsequent polymerase chain reaction using the OneStep RT-PCR kit (Qiagen). Primers capable to coamplify GLUT1 and GLUT4 cDNAs were used (Table 1), which allowed resolving their respective products on the basis of a 12-base-pair size difference [23]. Cycling conditions were as follows: 30 min at 50 °C for reverse transcription, 15 min at 95 °C for reverse transcriptase inactivation and polymerase activation followed by 25 cycles of 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C. After a final extension step of 10 min at 72 °C, PCR products were resolved by electrophoresis on 10% polyacrylamide gels in 1X TBE (89 mM Tris–borate, 2 mM EDTA) buffer. The gels were stained with ethidium bromide and a fluorescent image acquired with a digital camera. Quantitative analysis of bands on gel was done with the NIH Image 1.62 software.

For quantitative real-time RT-PCR, total RNA (100 ng) was treated with DNase I (Invitrogen) and reverse transcribed with the SuperScript II reverse transcriptase (Invitrogen). After degradation of the RNA with RNase H (Invitrogen), the cDNA was used for real-time polymerase chain reactions set up with the iQ Supermix kit (Bio-Rad) and run on an iCycler thermal cycler (Bio-Rad). Cycling conditions were as follows: 2.5 min at 95 °C for polymerase activation followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Detection of PCR products, based on the TaqMan technology, was performed by the iCycler iQ detection and software system (Bio-Rad). Primers and probes sequences are given in Table 1. The expression of GLUT4 was normalized for that of the housekeeping gene cyclophilin.

2.7. Immunofluorescence

ARC cultured on chamber slides were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.3% Triton X-100 in PBS. Primary antibodies used were directed against sarcomeric α-actinin (Sigma A7811), α-smooth muscle actin (Sigma A2547), ANF (Peninsula Laboratories T-4015) or MEF2 (Santa Cruz Biotechnology sc-313). Fluorophore-conjugated secondary antibodies were anti-mouse IgG-FITC (Sigma F5262) and anti-rabbit IgG-rhodamine (Chemicon AQ132R). All antibodies were diluted in PBS containing 1.5% non-immune goat serum. Counterstaining for F-actin was obtained with either rhodamine-phalloidin (Molecular Probes, Eugene, OR) or FITC-phal-

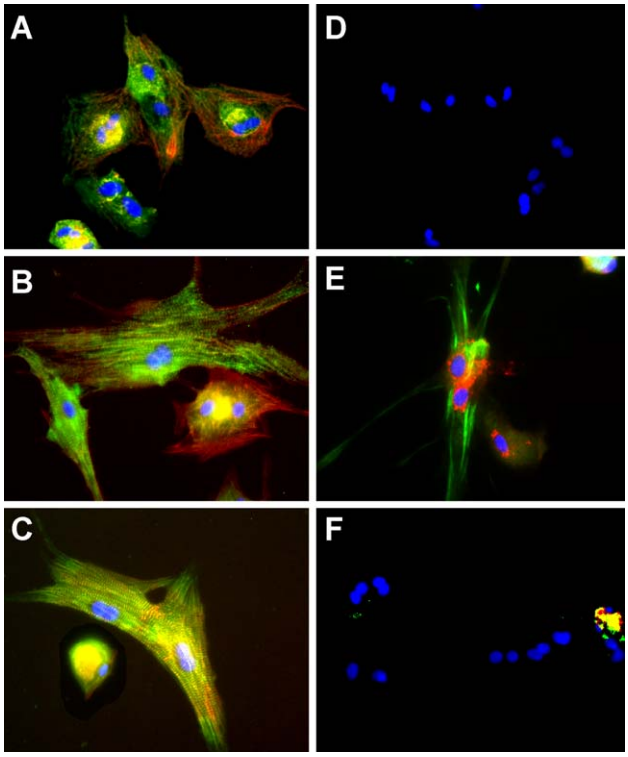

Fig. 1. Changes in cell phenotype induced by FGF-2 and IGF-1. ARC were cultured for 7 days in medium containing the vehicle (A, D), 25 ng/ml FGF-2 (B, E) or 500 ng/ml IGF-1 (C, F). ARC were fixed and stained for F-actin (red) and α-sarcomeric actinin (green) in panels A, B and C or for ANF (red) and α-smooth muscle actin (green) in panels D, E and F. Note that in panel C, because of the great difference in cell thickness, the image of each cell was processed separately.

Table 1
RT-PCR primers and probes

Duplex RT-PCR [23]	
GLUT1/4	Forward: 5′-GTCATCAACGCCCC(A/C)CAGAA-3′ Reverse: 5′-GAGAAGATGGCCACGGAGAGAG-3′
Quantitative real-time RT-PCR [28]	
GLUT1	Forward: 5′-CATCGTCGTTGGGATCCTTA-3′ Reverse: 5′-GAGACAGTAGAGGCCACAAGTCT-3′ Probe: 5′-FAM-AGGTGTTCGGCTTAGACTCCATCATGG-TAMRA-3′
GLUT4	Forward: 5′-CCCCCGATACCTCTACAT-3′ Reverse: 5′-GCATCAGACACATCAGCCCAG-3′ Probe: 5′-FAM-CTGCCCGAAAGAGTCTAAAGCGCCT-TAMRA-3′
Cyclophilin	Forward: 5′-CTGATGGCGAGCCCTTG-3′ Reverse: 5′-TCTGCTGTCTTTGGAACTTTGTC-3′ Probe: 5′-FAM-CGCGTCTGCTTCGAGCTGTTTGCA-TAMRA-3′

loidin (Sigma). DNA was stained with DAPI (Molecular Probes). Slides were mounted with VectaShield (Vector Laboratories) and examined with a Carl Zeiss Axiophot I microscope equipped with an Axiocam color CCD camera (Carl Zeiss). Images were acquired with the AxioVision software (Carl Zeiss) and processed with Photoshop 3.0.5 (Adobe Systems).

2.8. Electrophoretic mobility shit assay (EMSA)

For nuclear proteins extraction, ARC in 10-cm dishes were scraped in 2 ml ice-cold hypotonic buffer (20 mM HEPES pH 7.5, 10 μM Na_2MoO_4, 0.1 μM EDTA, protease and phosphatase inhibitors) and left on ice for 10 min. Igepal CA-630 was then added to a final concentration of 0.5% v/v and the extract was spun at 13000 rpm for 30 s to pellet the nuclei. The pellet was resuspended in 40 μl complete lysis buffer (20 mM HEPES pH 7.5, 400 mM NaCl, 20% glycerol, 1 mM DTT, 10 μM Na_2MoO_4, 0.1 μM EDTA, protease and phosphatase inhibitors) and kept on ice for 30 min with intermittent shaking. The nuclear extract was then spun at 13,000 rpm for 10 min and the supernatant containing nuclear proteins saved and stored at −80° C until EMSA was performed.

The rat *glut4* promoter MEF2 probe was constructed by annealing shifted oligonucleotides (sense 5′-GCCTAACG-TGGGAGCTAAAAATAGCCATTCCGG-3′; antisense 3′-TGCACCCTCGATTTTTATC GGTAAGGCCCATG-5′; MEF2 consensus sequence underlined) and filling in by the Klenow reaction in the presence of [α-^{32}P]CTP [24]. Nuclear proteins (15 μg) were incubated with 50,000 cpm MEF2 probe in complete lysis buffer for 30 min at room temperature in presence or absence of a 250-fold excess of

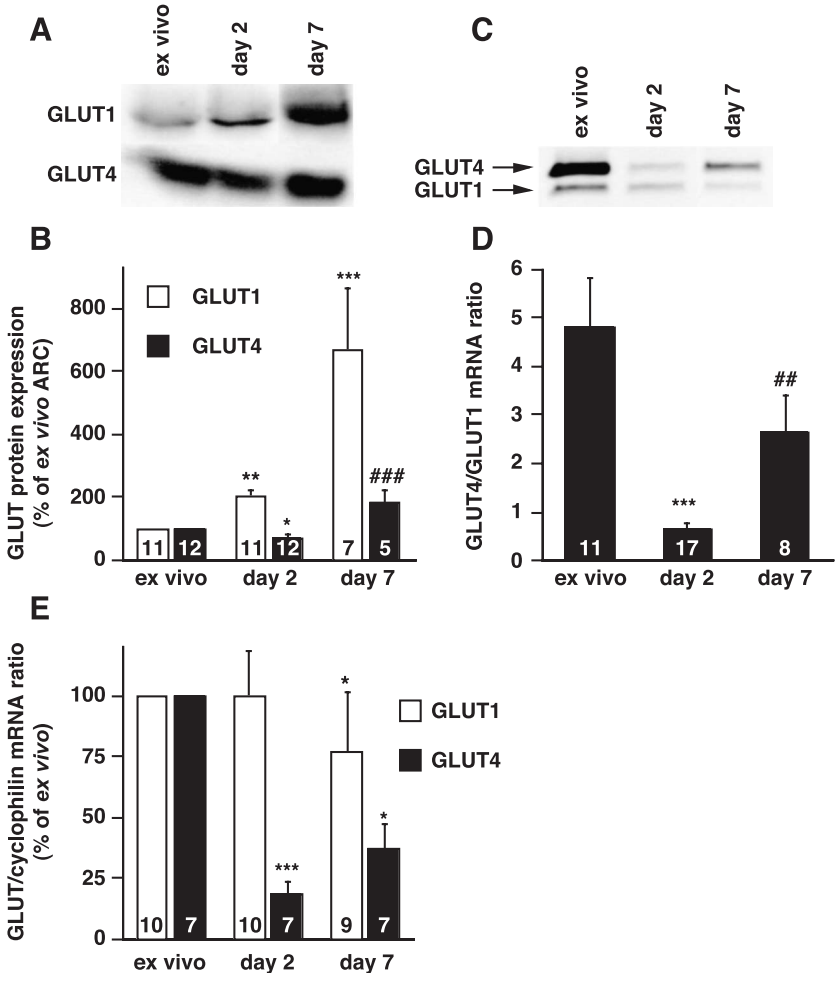

Fig. 2. ARC in primary culture spontaneously upregulate GLUT1 and downregulate GLUT4. (A) Representative immunoblots of GLUT1 and GLUT4 in ex vivo ARC or ARC after 2 and 7 days in primary culture. (B) Quantitative analysis of GLUT1 and GLUT4 proteins expression in ex vivo ARC (set at 100%) or ARC after 2 and 7 days in primary culture. (C) Representative RT-PCR of GLUT1 and GLUT4 mRNA in ex vivo ARC or ARC after 2 and 7 days in primary culture. (D) GLUT4/GLUT1 mRNA molar ratio in ex vivo ARC or ARC after 2 and 7 days in primary culture. (E) Expression of GLUT4 mRNA was measured by quantitative RT-PCR and expressed as percent of the value in ex vivo ARC. Results are mean ± S.E.M. *$p<0.05$; **$p<0.01$; ***$p<0.001$ vs. ex vivo. $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ vs. day 2.

unlabeled probe and the complexes were resolved on 5% non denaturing polyacrylamide gels [24].

2.9. Statistics

Statistical analyses were performed with the Prism4 software (GraphPad Softwares). Multiple groups were compared by the Kruskal–Wallis test, followed by Dunn's post-hoc tests for pair wise comparison. When two treatments were combined (i.e. agonist and inhibitor) results were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. The number of experiments or cells analyzed is indicated at the foot of each bar in bar graphs. Differences were considered significant when p was less than 0.05.

3. Results

3.1. Spontaneous modification of ARC phenotype and effect of FGF-2 and IGF-1

Two days following plating of the ARC, cells were either rod-shaped or rounded up by hypercontraction, with only minimal cell spreading. No differences were apparent between control and growth factor-treated ARC. After 7 days in culture, ARC had started to disassemble the contractile apparatus and spread onto the substratum. A "myofibrillar ball" persisted in the perinuclear region (Fig. 1A). To study the influence of growth factors that have been shown to promote or attenuate cellular features of dedifferentiation [15], ARC were cultured in medium containing, respectively, FGF-2 or IGF-1. In the presence of FGF-2, ARC were much larger, with myofibrils restricted to a central perinuclear region (Fig. 1B). Fig. 1C shows two ARC cultured with 500 ng/ml IGF-1. One cell is fully spread and shows extensive myofibrillar organization, while the other essentially shows a "myofibrillar ball". In contrast to control and IGF-1-treated ARC (Fig. 1D,F), ARC exposed to FGF-2 exhibited expression of the markers of myocyte hypertrophy and dedifferentiation ANF and α-smooth muscle actin. ANF was visible in perinuclear granules (rhodamine label), while α-smooth muscle actin was seen in stress fiber-like structures (FITC label).

3.2. GLUT1 and GLUT4 expression during spontaneous modification of phenotype of ARC

ARC in long-term culture exhibited spontaneous modifications of expression of the glucose transporter proteins GLUT1 and GLUT4. After 2 days in primary culture, the protein content of GLUT1 was markedly increased compared with the value measured in ARC immediately after isolation (thereafter referred to as ex vivo ARC). Concomitantly, the protein content of GLUT4 was decreased (Fig. 2A,B). Coamplification of GLUT1 and GLUT4 mRNA by RT-PCR using a common set of primers revealed a marked drop in the GLUT4/GLUT1 mRNA molar ratio in ARC after 2 days in primary culture as compared to ex vivo ARC (Fig. 2C,D). Seven days after isolation, GLUT1 protein expression was further increased, while expression of GLUT4 was restored to levels even above ex vivo levels. Consistent with restoration of GLUT4 expression, the GLUT4/GLUT1 mRNA molar ratio also increased and reached almost ex vivo levels. Expression of GLUT4 mRNA as measured by quantitative RT-PCR followed a pattern parallel to that of the GLUT4/GLUT1 mRNA ratio (Fig. 2E), whereas expression of GLUT1 mRNA did not change significantly after 2 days and decreased slightly after 7 days.

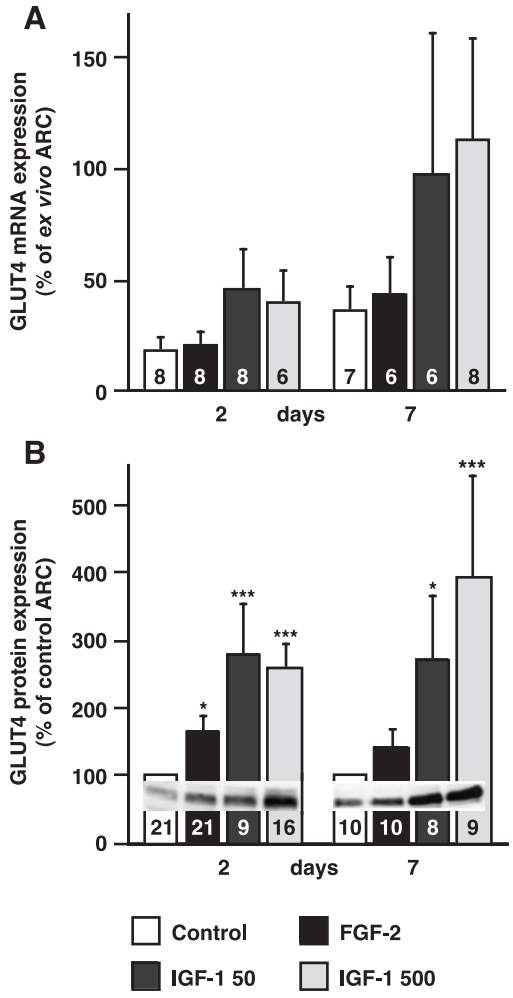

Fig. 3. FGF-2 and IGF-1 upregulate GLUT4 expression. ARC were plated in medium containing 25 ng/ml FGF-2, 50 ng/ml IGF-1, 500 ng/ml IGF-1 or the vehicle. After indicated times, total RNA and proteins were extracted. (A) Expression of GLUT4 mRNA was measured by quantitative RT-PCR and expressed as percent of the value in ex vivo ARC.
(B) Expression of the GLUT4 protein was determined by immunobloting. Results are mean ± S.E.M. *$p<0.05$; **$p<0.01$; ***$p<0.001$ vs. control. Insets show representative Western blots.

3.3. Regulation of GLUT4 expression by FGF-2 and IGF-1

IGF-1 induced a marked increase in GLUT4 protein expression on both day 2 and day 7. In contrast, FGF2 only slightly increased the expression of the GLUT4 protein (Fig. 3B).

Changes at the level of protein expression were paralleled by alterations of the GLUT4 mRNA expression. After 2 days, treatment with FGF-2 did not increase the level of the GLUT4 mRNA, whereas IGF-1 increased the GLUT4 mRNA (Fig. 3A). After 7 days of treatment with IGF-1, the expression of GLUT4 mRNA was increased more than twofold. Again, FGF-2 had no effect.

3.4. Signaling pathways involved in regulation of GLUT4 expression

To determine whether signaling pathways were activated by the isolation procedure itself, measurements in extracts of hearts obtained immediately after surgical excision (thereafter referred to as native hearts) were compared to ex vivo ARC and to ARC kept in culture for 4, 24 and 48 h. An obvious limitation of this approach is the comparison of a whole tissue homogenate of native hearts, containing several cell types, with a 95% pure ARC population. The ERK1/2 MAP kinase and the PI3K pathways were examined, because these signaling cascades are major determinants of GLUT1 [25] and GLUT4 expression [26,27]. In addition, we measured activation of the p38 MAPK cascade, which was likely to be activated in response to the stress of isolation.

Activity of the PI3K pathway, estimated from the phosphorylation level of the downstream kinase Akt on Ser-473, was not stimulated by isolation of the ARC, as the variations in pSer473-Akt observed between native hearts and ex vivo ARC paralleled the variations in total Akt protein. In fact, quantitative analysis indicated that activity of the PI3K pathway was reduced upon ARC isolation (Fig. 4A). In contrast, both the ERK1/2 and the p38 MAP kinase pathways showed marked activation immediately after isolation. Quantitative analysis of ERK1/2 and p38 MAPK activities revealed a 14- and 9-fold activation upon isolation, respectively. p38 MAPK activity returned to baseline within 2 days, whereas ERK1/2 activity remained elevated.

FGF-2 and IGF-1 elicited a further increase of activation of ERK1/2 and p38 MAPK 10 min after plating (Fig. 4B). In addition, IGF-1, but not FGF-2, strongly activated Akt, which had been inactivated upon isolation.

To elucidate the role of the role of ERK1/2, and p38 MAPK on growth factor-stimulated increase of GLUT4, ARC were cultured for 2 days with medium containing growth factors and selective inhibitors. Growth factor stimulation in the presence of an inhibitor was expressed relative to incubation with the inhibitor alone. It is of note that U0126 increased basal expression GLUT4 by 294%, while the p38 MAPK inhibitor SB203580 slightly increased basal GLUT4 expression. Fig. 5 depicts the effects of inhibitors on GLUT4 expression. U0126 did not significantly alter GLUT4 expression in ARC exposed to either FGF-2 or IGF-1. In contrast, SB203580 completely abolished the pronounced overexpression of GLUT4 induced by IGF-1 after both 2 (A) and 7 (B) days. Thus, increased protein expression of GLUT4 in response to IGF-1 seems to be largely mediated by p38 MAPK.

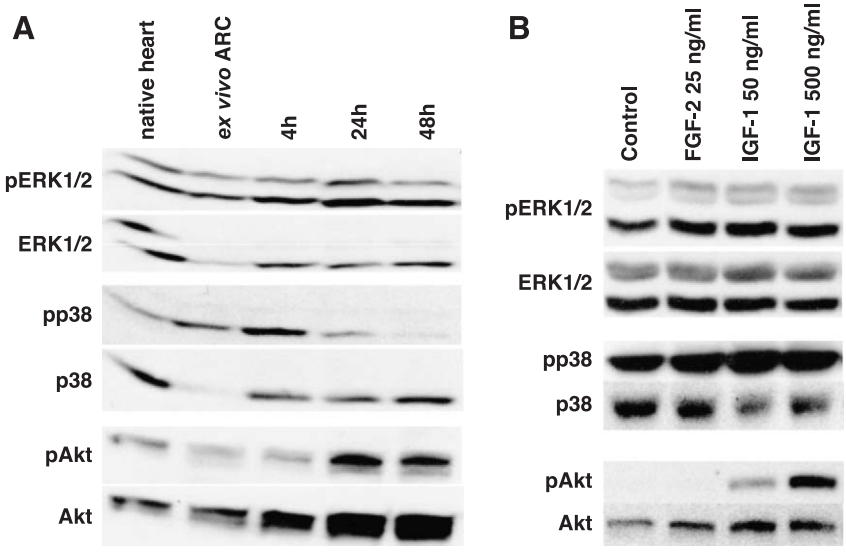

Fig. 4. Isolation and growth factors activate MAP kinase pathways. (A) Isolation activates ERK1/2 and p38 MAPK, but deactivates Akt. Representative immunoblots showing activated (phosphorylated) and total ERK1/2, p38 MAPK and Akt in native heart extracts, ex vivo ARC and ARC in culture for 4, 24 and 48 h. (B) Growth factors activate ERK1/2, p38 MAPK and Akt. Representative immunoblots showing activated (phosphorylated) and total ERK1/2, p38 MAPK and Akt in ARC treated with FGF-2 or IGF-1 for 10 min immediately after isolation.

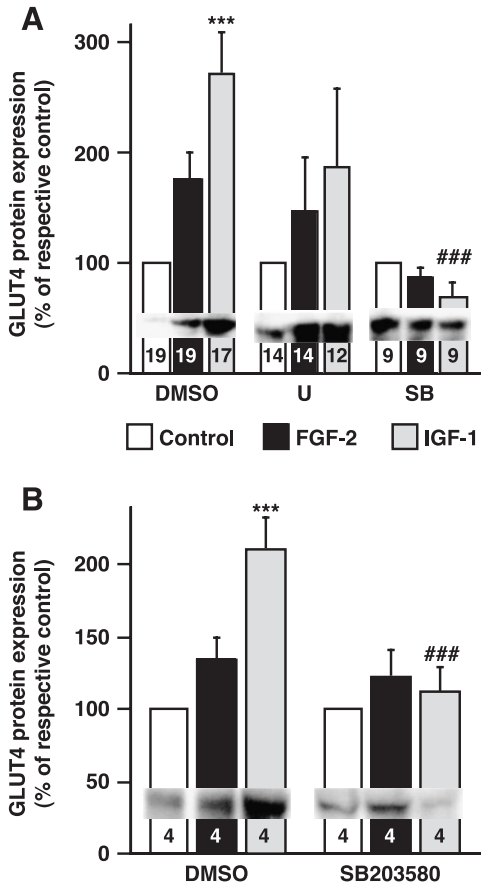

Fig. 5. Activation of the p38 MAPK pathway mediates upregulation of GLUT4. Quantitative analysis of GLUT4 protein expression in ARC after 2 days (A) and 7 days (B) in primary culture in presence of 0.1% DMSO, U0126 (U; 10 μM) or SB203580 (SB; 10 μM). ARC were also incubated with FGF-2, IGF-1 or no agonist. For each inhibitor, the results were normalized for expression in absence of agonist. Results are mean ± S.E.M. $^{**}p < 0.01$ vs. no agonist; $^{\#\#}p < 0.01$ vs. value with the same agonist but no inhibitor. Insets show representative Western Blots.

3.5. Effects of ERK1/2 and p38 MAPK on transcriptional activation of the GLUT4 promoter in neonatal rat cardiomyocytes

The effects of activation of ERK1/2 and p38 MAPK on transcriptional activity of the *glut4* promoters were investigated in neonatal rat cardiomyocytes (NRC). We resorted to NRC for these experiments because ARC are not amenable to conventional transfection techniques. As in ARC, IGF-1 increased the expression of the GLUT4 mRNA in NRC (Fig. 6A) in a p38 MAPK-dependent manner.

Transfection of the active ERK1/2 mutant DN3MEK1 did not significantly alter *glut4* promoter activity (Fig. 6B). In contrast, activation of p38 MAPK by MEK6DD elicited robust stimulation of *glut4* promoter activity, which was entirely blocked by SB203580 (Fig. 6C). Thus, p38 MAPK, but not ERK1/2, seems to be capable of stimulating *glut4* promoter activity.

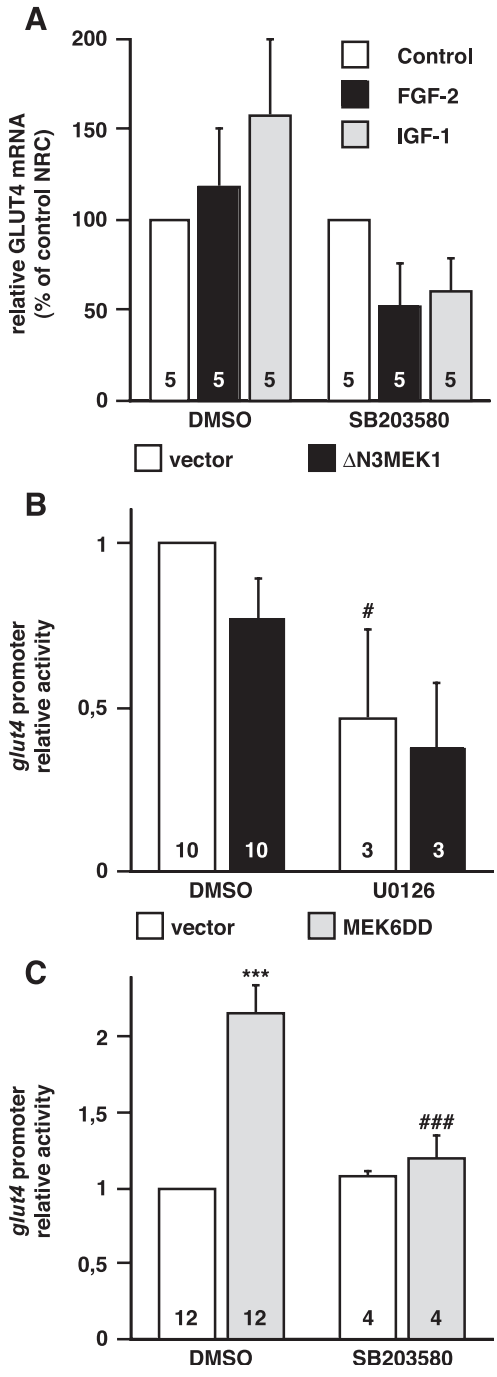

Fig. 6. Activation of the *glut4* promoter by the p38 MAPK pathways. (A) Quantitative analysis of GLUT4 mRNA expression in neonatal rat cardiomyocytes (NRC) in primary culture in presence of 0.1% DMSO or SB203580 (SB; 10 μM). ARC were also incubated with FGF-2, IGF-1 or no agonist. For each inhibitor, the results were normalized for expression in absence of agonist. (B) Relative luciferase expression driven by either the *glut4* promoter was determined in NRC cotransfected with expression plasmids encoding activated MEK1 (DN3MEK1; A), activated MEK6 (MEK6DD, B) or the empty respective vector plasmid. NRC were treated with the MEK1 inhibitor U0126 (10 μM), the p38 MAPK inhibitor SB203580 (10 μM) or the vehicle DMSO. Results are mean ± S.E.M. $^{***}p < 0.001$ vs. empty vector. $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ vs. NRC expressing the same activated MEK in absence of inhibitor.

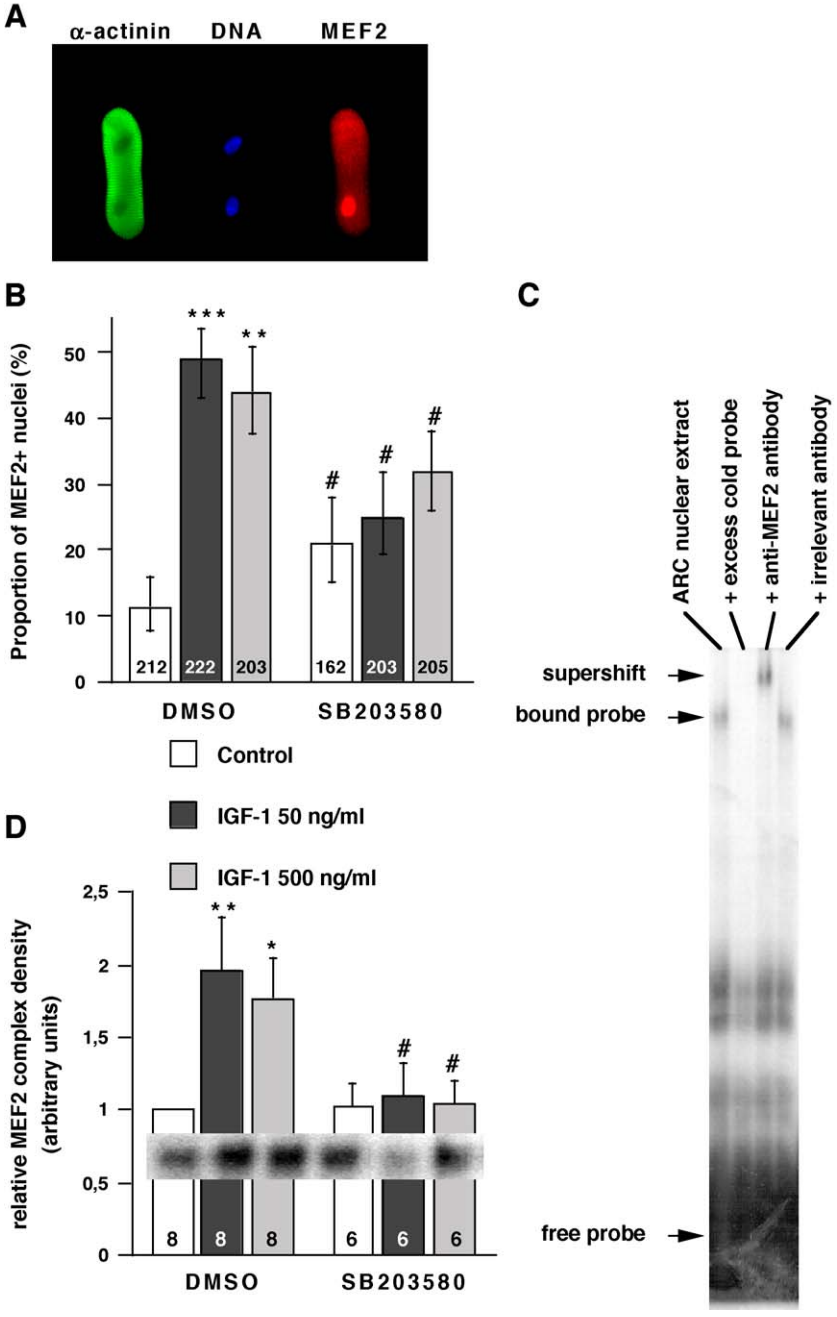

Fig. 7. MEF2 activation in ARC treated with growth factors. Fixed and permeabilized ARC were stained with anti-α-actinin antibody (green), DAPI (blue) and anti-MEF2 antibody (red). Nuclei were counted as positive if the MEF2 staining was evident against cytoplasmic background. (A) Example of an ARC with one MEF2-negative and one MEF2-positive nucleus. (B) Proportions of MEF2-positive nuclei in ARC treated for 2 days with 50 ng/ml IGF-1, 500 ng/ml IGF-1 or the vehicle in the presence or absence of the p38 MAPK inhibitor SB203580 (10 μM). Error bars indicate the 95% confidence interval of the proportion. $^{**}p<0.01$; $^{***}p<0.001$ for IGF-1 treatment vs. control. $^{\#}p<0.05$ for SB203580 treatment vs. no inhibitor. (C) Representative electrophoretic mobility shift assay showing a single specific MEF2 complex, identified by competition with excess cold probe and by supershift with an anti-MEF2 antibody. (D) Quantitation of MEF2 complexes in nuclear extracts from ARC treated with 50 ng/ml IGF-1, 500 ng/ml IGF-1 or the vehicle in the presence or absence of the p38 MAPK inhibitor SB203580 (10 μM). Inset shows a representative experiment. $^{*}p<0.05$, $^{**}p<0.01$ for IGF-1 treatment vs. vehicle. $^{\#}p<0.05$ for SB203580 treatment vs. DMSO.

3.6. Role of MEF2 in the regulation of GLUT4 expression

We tested the hypothesis that, downstream of p38 MAPK, the transcription factor MEF2 mediated the overexpression of GLUT4 in response to IGF-1. Nuclear retention of MEF2 in fixed permeabilized ARC was used as an index of increased DNA affinity. The percentage of MEF2-positive nuclei (i.e. MEF2 staining showing up in the nucleus against cytoplasmic background, Fig. 7A) was low in control ARC after 2 days (11% of MEF2-positive

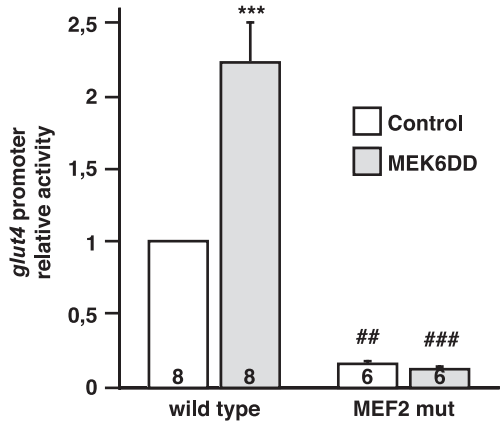

Fig. 8. Activation of the *glut4* promoter by the p38 MAPK pathway is MEF2-dependent. Relative luciferase expression driven by either the wild-type *glut4* promoter or the *glut4* promoter mutated at the MEF2 binding site (MEF2mut) was determined in NRC cotransfected with an expression plasmid encoding activated MEK6DD or the empty vector plasmid. Results are mean ± S.E.M. ***$p<0.001$ vs. empty vector. $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ MEF2mut vs. wild-type *glut4* promoter.

nuclei, 95% confidence interval 8–16%, $n=212$), but increased after 7 days in parallel with reexpression of GLUT4 (39% of MEF2-positive nuclei, 95% confidence interval 32–47%, $n=166$). Both dosages of IGF-1 increased the proportion of MEF2-positive nuclei, an effect that was blunted by inhibition of p38 MAPK activity with SB20358 (Fig. 7B). Electrophoretic mobility shift assays using the MEF2 consensus sequence from the rat *glut4* promoter showed a single specific complex of ARC nuclear proteins with the *glut4* MEF2 probe, identified by competition with excess cold probe and by supershift with an anti-MEF2 antibody (Fig. 7C). The intensity of the MEF2 complex was greater in nuclear extracts from IGF-1-treated ARC; again, inhibition of p38 MAPK activity reduced binding ARC nuclear proteins to the *glut4* MEF2 probe (Fig. 7D).

To further confirm the importance of MEF2 for p38 MAPK-stimulated GLUT4 transcription, we mutated the MEF2 consensus sequence in the reporter plasmid pLucGT4 used in transient transfection experiments in NRC. Basal transcription from the mutated *glut4* promoter was markedly reduced as compared with the wild-type promoter (Fig. 8). In addition, transcription from the mutated *glut4* promoter was totally insensitive to expression of MEK6DD, indicating that MEF2 binding activity is required for transduction of the p38 MAPK signals to the *glut4* promoter.

4. Discussion

Cardiac hypertrophy and failure is associated with downregulation of GLUT4, which potentially contributes to altered myocardial structure, metabolism and function [11,12]. The results of the present study in ARC indicate that IGF-1 can restore GLUT4 expression through p38 MAPK-mediated activation of MEF2.

4.1. ARC differentiation and GLUT expression

One of the aims of this study was to examine the relationship between the state of differentiation of cardiac myocytes and the relative expression of GLUT isoforms, taking advantage of the dedifferentiation–redifferentiation ARC model and of the possibility to manipulate the degree of differentiation. The results demonstrated that isolation of ARC in itself elicited not only morphological dedifferentiation of the myocytes, but also corresponding changes in the mRNA and protein expression of GLUT isoforms. Freshly isolated (ex vivo) ARC had a GLUT4/GLUT1 mRNA molar ratio of 4.8, a value similar to that reported for native cardiac tissue (3.8) [23]. After 2 days in primary culture, this ratio had dropped to 0.65, a value below that reported for neonatal rat cardiac myocytes (0.84) [25]. During the subsequent redifferentiation process, GLUT4 protein expression was increased after 7 days and the GLUT4/GLUT1 mRNA molar ratio was reaugmented to 2.7. Concomitantly with the effects of FGF-2 and IGF-1 on markers of dedifferentiation such as ANF, α-smooth muscle-actin and myofibrillar organization [14–16], IGF-1 more potently than FGF-2 restored expression of GLUT4 towards ex vivo values. This suggests that the GLUT isoform expression pattern may provide an index of differentiation of cardiac myocytes. Indeed, similar dysregulation of glucose transporters expression were observed in pathological conditions challenging cardiac myocytes differentiation such as hypertrophy, unloading [28] or failure [9]. The reason why the GLUT1 protein expression increased whereas the GLUT1 mRNA remained stable or even slightly decreased remains unknown.

4.2. Signal transduction and regulation of GLUT expression

The marked upregulation of GLUT4 observed in ARC in response to IGF-1 was completely abolished by the p38 MAPK inhibitor SB203580. This strongly suggests that p38 MAPK is the main effector of GLUT4 upregulation in ARC in primary culture. Consistent with the effects of inhibition of ERK1/2 and p38 MAP kinases on GLUT4 expression in ARC, transfection experiments in NCR indicated that p38 MAPK but not ERK1/2 activate transcription from the *glut4* promoter. Such a positive regulation of GLUT4 by p38 MAPK has to the best of our knowledge not been reported. It should be emphasized that FGF-2 and IGF-1 activated p38 MAPK to a similar extent, while the pronounced upregulation of GLUT4 was observed only in the presence of IGF-1, and only to a small extent in the presence of FGF-2.

We used a comparatively high dose of IGF-1 (500 ng/ml) based on previous studies examining the differential effects of FGF-2 and IGF-1 in the ARC model [15]. A potential concern with such a high dose of IGF-1 is activation of the insulin receptor leading to effects that are not specific for IGF-1. We obtained results that were almost identical when

we used a 10-fold lower dose of IGF-1 (50 ng/ml, Fig. 3). We do not believe that activation of the insulin receptor played a significant role in our observations as even the high dose of IGF-1 (500 ng/ml or ca. 66 nM) was below the estimated K_d for activation of the insulin receptor by IGF-1 (100–500 nM) [29].

Transcription factors of the Myocyte Enhancer Factor 2 family are required for expression of GLUT4 in adipocytes and both heart and skeletal muscle [30,31]. MEF2 is known to be activated by p38 MAPK [32]. Our results clearly indicate that IGF-1 activated MEF2 in a p38 MAPK-dependent manner and that MEF2 activation was required for increased expression of GLUT4 in response to IGF-1. More nuclei were MEF2-positive in IGF-1-treated ARC, which indirectly suggests increased affinity of MEF2 for DNA, preventing diffusion of MEF2 out of the nuclei of fixed and permeabilized cells. A similar interpretation was proposed for the glucocorticoid receptor [33]. This conclusion was confirmed by more specific electrophoretic mobility shift assays measuring the binding of ARC nuclear proteins to a MEF2 consensus sequence derived from the rat *glut4* promoter. An interesting observation in this context is that TGF-β inhibits differentiation of myogenic cells through translocation of MEF2 from the nucleus to the cytoplasm [34]. It may be speculated that the mechanism(s) that triggered dedifferentiation of ARC similarly induced translocation of MEF2 to the cytoplasm.

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