The Transforming Growth Factor-β Superfamily Member Growth-Differentiation Factor-15 Protects the Heart From Ischemia/Reperfusion Injury

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Abstract—Data from the Women’s Health Study show that serum levels of growth-differentiation factor-15 (GDF-15), a distant member of the transforming growth factor-β superfamily, are an independent risk indicator for adverse cardiovascular events. However, the cellular sources, upstream regulators, and functional effects of GDF-15 in the cardiovascular system have not been elucidated. We have identified GDF-15 by cDNA expression array analysis as a gene that is strongly upregulated by nitrosative stress in cultured cardiomyocytes isolated from 1- to 3-day-old rats. GDF-15 mRNA and pro-peptide expression levels were also induced in cardiomyocytes subjected to simulated ischemia/reperfusion (I/R) via NO–peroxynitrite-dependent signaling pathways. GDF-15 was actively secreted into the culture supernatant, suggesting that it might exert autocrine/paracrine effects during I/R. To explore the in vivo relevance of these findings, mice were subjected to transient or permanent coronary artery ligation. Myocardial GDF-15 mRNA and pro-peptide abundance rapidly increased in the area-at-risk after ischemic injury. Similarly, patients with an acute myocardial infarction had enhanced myocardial GDF-15 pro-peptide expression levels. As shown by immunohistochemistry, cardiomyocytes in the ischemic area contributed significantly to the induction of GDF-15 in the infarcted human heart. To delineate the function of GDF-15 during I/R, Gdf-15 gene-targeted mice were subjected to transient coronary artery ligation for 1 hour followed by reperfusion for 24 hours. Gdf-15−deficient mice developed greater infarct sizes and displayed more cardiomyocyte apoptosis in the infarct border zone after I/R compared with wild-type littersmates, indicating that endogenous GDF-15 limits myocardial tissue damage in vivo. Moreover, treatment with recombinant GDF-15 protected cultured cardiomyocytes from apoptosis during simulated I/R as shown by histone ELISA, TUNEL/Hoechst staining, and annexin V/propidium iodide fluorescence-activated cell sorting (FACS) analysis. Mechanistically, the prosurvival effects of GDF-15 in cultured cardiomyocytes were abolished by phosphoinositide 3-OH kinase inhibitors and adenoviral expression of dominant-negative Akt1 (K179M mutation). In conclusion, our study identifies induction of GDF-15 in the heart as a novel defense mechanism that protects from I/R injury. (Circ Res. 2006;98:351-360.)

Key Words: growth-differentiation factor-15 ▪ ischemia/reperfusion ▪ apoptosis ▪ PI3K ▪ Akt

Coronary reperfusion is the primary therapeutic goal in patients with acute myocardial infarction (AMI). Although reperfusion is essential for myocardial salvage, it may at first exacerbate cellular damage sustained during the ischemic period, a phenomenon known as reperfusion injury.1 There is growing evidence that the myocardium adapts to ischemia/reperfusion (I/R) by synthesizing and responding to a variety of stress-induced growth factors and cytokines, and that identification of these endogenous homeostatic mechanisms may open new avenues to limit I/R injury.2,3

Transforming growth factor-βs (TGF-βs) constitute a superfamily of cytokines that exert prominent functions in adult tissue homeostasis and adaptation by regulating cell survival, proliferation, and differentiation. Increases or decreases in the production of TGF-βs have been linked to a number of disease states, including neurodegenerative disorders and atherosclerosis.4 Growth-differentiation factor-15 (GDF-15), which is identical to macrophage-inhibitory cytokine-1, placent al bone morphogenetic protein, placental transforming growth factor-β, and nonsteroidal antiinflammatory drug-ac-
tivated gene-1, is a distant member of the TGF-β superfamily.\textsuperscript{5–7} GDF-15 is produced as a \textasciitilde 40-kDa pro-peptide monomer, which is processed to a mature \textasciitilde 30-kDa secreted peptide.\textsuperscript{5} Cell culture experiments suggest that GDF-15 can act as a neuronal survival factor.\textsuperscript{6} Conversely, GDF-15 promotes cell death in a number of tumor cell lines,\textsuperscript{8,9} indicating a role for GDF-15 in the execution of cell death or cell survival programs. Data from the Women’s Health Study show that GDF-15 serum levels are an independent risk indicator for adverse cardiovascular events, including stroke and AMI.\textsuperscript{11} However, the cellular sources, upstream regulators, and functional effects of GDF-15 in the cardiovascular system have not been elucidated. In fact, no study has ever assigned a specific in vivo function to GDF-15.

We have identified GDF-15 by cDNA expression array analysis as a gene that is massively upregulated by nitrosative stress in cardiomyocytes subjected to simulated I/R. In vivo, we found GDF-15 to be strongly induced in the infarcted murine myocardium and in left ventricular (LV) tissue samples obtained from patients who had died after an AMI. Using Gdf-15 gene-targeted mice,\textsuperscript{7} we demonstrate that endogenous GDF-15 protects the heart from I/R injury. In cell culture, recombinant GDF-15 protects cardiomyocytes from ischemic injury via phosphoinositide 3-OH kinase (PI3K) and Akt-dependent signaling pathways. Our results identify GDF-15 as a novel cardioprotective cytokine.

**Materials and Methods**

For an extended Materials and Methods section, please refer to the online data supplement, available at http://circres.ahajournals.org.

**Materials**

Human GDF-15, rat leukemia inhibitory factor (LIF), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) were purchased from R&D Systems, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazide (AMT), N(G)-nitro-L-arginine methyl-ester (L-NAME), and tetramethylammonium-peroxynitrite from Alexis, Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP) from Calbiochem, and 8-para-chlorophenylthio-cGMP (8-pCPT-cGMP) from Biolog.

**Cardiomyocyte Culture**

Ventricular cardiomyocytes were isolated from 1- to 3-day-old Sprague-Dawley rats (Charles River, Germany) and exposed to simulated ischemia or I/R.\textsuperscript{12} Cell death was assessed by lactate dehydrogenase (LDH) release, histone ELISA, in situ TUNEL/Hoechst 33258 staining, and annexin V/propidium iodide (PI) staining and FACS analysis. Where shown, cardiomyocytes were infected with a replication-deficient adenovirus encoding dominant-negative Akt1 (K179M mutation, Ad.dnAkt1, kindly provided by Dr Richard Patten, Tufts-New England Medical Center, Boston, Mass).\textsuperscript{13}

**Surgical Procedures**

Eight- to 12-week-old male C57BL/6 mice were subjected to permanent or transient left anterior descending coronary artery (LAD) ligation or a sham operation. Gdf-15 gene-targeted mice were

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** GDF-15 is induced by nitrosative stress in cardiomyocytes. Cardiomyocytes were cultured for 24 hours in the absence or presence of SNAP (250 μmol/L), MnTBAP (100 μmol/L), peroxynitrite (100 μmol/L), 8-pCPTcGMP (500 μmol/L), IL-1β (10 ng/mL), IFN-γ (50 ng/mL), or AMT (100 μmol/L). GDF-15 mRNA and 18S rRNA expression levels were quantified by Northern blot (A, D, and E). Cell lysates and culture supernatants (1 mL per condition) were processed for GDF-15 and α-actinin immunoblotting analysis (B and C). A time course is shown (E). Data from n = 3 to 6 experiments are presented (D and E). *P < 0.05, **P < 0.01 vs control; #P < 0.01 vs SNAP or IL-1β/IFN-γ alone.
kindly provided by Dr Se-Jin Lee (Johns Hopkins University, Baltimore, Md) and maintained on a C57BL/6J/129/SvJ background. Eight- to 16-week-old male and female Gdf-15–deficient mice and their wild-type (WT) littersmates underwent transient LAD ligation. Area at risk and infarct sizes were determined by Evans blue and 2,3,5-triphenyltetrazolium chloride (TTC) staining. Apoptotic cardiomyocytes in the infarct border zone were detected by TUNEL/Hoechst staining and anti–a-actinin immunostaining. Heart rate, mean arterial blood pressure, and maximal LV pressure were determined in a closed chest preparation using a 1.4 F Millar catheter advanced via the right carotid artery into the ascending aorta and left ventricle.

Human Myocardial Tissue
LV tissue samples were collected at autopsy from 17 patients who had died from an AMI (10 males; mean age 66±3 years) and from 5 patients who had died from noncardiac causes (2 males; mean age 59±9 years). Viable and reversibly damaged (jeopardized) myocardial areas were discriminated by anti-complement C3d staining.14

Statistical Analysis
Data are presented as means±SEM. Differences between groups were analyzed by one-way ANOVA followed by Student-Newman–Keuls post hoc test. A two-tailed P value <0.05 was considered to indicate statistical significance.

Results
Identification of GDF-15 as an NO-Regulated Gene in Cardiomyocytes
Expanding on our previous efforts to identify NO-regulated genes in cardiomyocytes that are functionally important,15,16 we performed cDNA expression array analyses in cardiomyocytes cultured for 24 hours in the presence or absence of the NO donor SNAP (250 μmol/L). Although this is still a sublethal concentration, higher concentrations of SNAP (eg, 1 mmol/L) promote overt cell death in our system. In two experiments, we found GDF-15 to be the gene that was induced most strongly by SNAP among 3906 genes represented on the Atlas Plastic Rat 4K Microarray (17-fold and 28-fold induction, respectively). This finding was confirmed by Northern blot analysis (Figure 1A). Increased mRNA levels were translated into increased GDF-15 pro-peptide levels and resulted in the release of the mature GDF-15 peptide into the culture supernatant (Figure 1B). Induction of GDF-15 by SNAP was evident within 4 hours, expression levels remained high after 12 to 24 hours, and returned to baseline after 48 hours (Figure 1C).

In general, NO can alter gene expression via cGMP-dependent and cGMP-independent signaling pathways. One important cGMP-independent pathway involves the reaction of NO with superoxide to form peroxynitrite.18 The effects of SNAP on GDF-15 mRNA abundance were reversed by the superoxide dismutase mimetic MnTBAP; conversely, peroxynitrite, when directly added to the cells, significantly increased GDF-15 expression levels (Figure 1D). In contrast, the cell-permeable cGMP analog 8-pCPT-cGMP did not induce GDF-15 expression in cardiomyocytes (Figure 1D). Together, induction of GDF-15 by NO appears to be mediated via superoxide/peroxynitrite-dependent but cGMP-independent pathways.

GDF-15 was also induced in cardiomyocytes treated with IL-1β/IFN-γ (Figure 1E), cytokines known to enhance ex-
pression of inducible NO synthase (NOS2) in cultured cardiomyocytes. Coincubation with the NOS2 inhibitor AMT or MnTBAP partially reversed the effects of IL-1β/IFN-γ on GDF-15 abundance, indicating that IL-1β/IFN-γ induce GDF-15 via endogenous NOS2 and generation of nitrosative stress (Figure 1E).

Cardiomyocytes Express and Secrete GDF-15 After Simulated Ischemia and I/R

Considering that NOS2 is activated and that NO and peroxynitrite are produced in the heart after ischemic injury, we explored whether GDF-15 is induced in cardiomyocytes subjected to simulated ischemia or I/R in vitro. As shown in Figure 2A, GDF-15 pro-peptide levels gradually increased during simulated ischemia, reaching maximum levels after 6 hours and returning toward baseline after 12 hours (later time points could not be studied because of severe cell damage); at the same time, mature GDF-15 was secreted and accumulated in the culture supernatant (Figure 2A). GDF-15 pro-peptide levels also increased after simulated I/R (Figure 2B). A certain period of ischemia appeared to be required for GDF-15 expression and secretion during reperfusion (eg, GDF-15 was not induced by 1 hour of ischemia followed by 5 hours of reperfusion but strongly expressed after 3 hours of ischemia followed by 3 hours of reperfusion). Cardiomyocytes that were reperfused after a 3-hour episode of simulated ischemia continued to express and secrete GDF-15 for up to 24 hours (Figure 2B). Treatment of cardiomyocytes with the NOS inhibitor L-NAME, AMT, or MnTBAP blunted the induction of GDF-15 during simulated I/R, indicating that NOS2-NO-peroxynitrite–dependent signaling pathways are involved (Figure 2C).

Induction of GDF-15 in the Murine Heart After Ischemia and I/R

To investigate whether GDF-15 is induced by ischemia or I/R in vivo, mice were subjected to permanent or transient LAD ligation (Figure 3A through 3C). Permanent coronary artery ligation resulted in a rapid induction of GDF-15 expression in the ischemic area; mRNA levels were upregulated within 1 hour (Figure 3A) and pro-peptide levels within 5 hours (Figure 3C). With time, GDF-15 mRNA and pro-peptide expression continued to increase in the ischemic area and remained elevated for at least 7 days. Similarly, coronary reperfusion after a 1-hour period of ischemia resulted in a progressive increase of GDF-15 expression in the area at risk (Figure 3A and 3C). The magnitude and kinetics of GDF-15 induction after ischemia or I/R were roughly comparable (eg, 5 or 24 hours of ischemia resulted in similar GDF-15 expression levels compared with 1 hour of ischemia followed by reperfusion for 4 or 24 hours, respectively). GDF-15 mRNA levels also increased in the remote left ventricle during permanent ischemia; however, this effect was less pronounced and transient (Figure 3A).

Increased Myocardial GDF-15 Expression Levels in Patients With AMI

GDF-15 pro-peptide expression levels were significantly increased in tissue samples obtained from the infarcted...
myocardium of patients who had died after an AMI (Figure 4A). GDF-15 expression increased within 12 hours of symptom onset and remained upregulated for at least 2 weeks (Figure 4B and 4C). Patients who had or had not received reperfusion therapy displayed similar GDF-15 expression levels (Figure 4B). As shown by immunohistochemistry, cardiomyocytes within irreversibly damaged myocardial areas strongly expressed GDF-15 (Figure 4Da). Irreversibly damaged and viable myocardial areas were distinguished by anti-complement C3d staining of a parallel section (Figure 4Db).14 No GDF-15 staining was detected when the primary antibody was omitted from the reaction (Figure 4Dc). GDF-15 was barely detectable in viable myocardial areas (compare Figure 4Da and 4Db) or in myocardial sections obtained from control patients (Figure 4Dd). The specificity of the anti-human GDF-15 antibody used during these studies was confirmed by immunoblotting of human LV myocardial protein extracts (Figure 4A).

Endogenous GDF-15 Protects the Heart From I/R Injury

To explore the functional effects associated with GDF-15 induction in the infarcted heart, Gdf-15–deficient (knockout [KO]) mice did not differ from WT mice with regard to their baseline heart weight-to-body weight ratio,21 heart rate (WT 387±36 minutes−1; KO 366±25 minutes−1), mean arterial pressure (WT 81±9 mm Hg; KO 75±5 mm Hg), and maximal LV pressure (WT 99±8 mm Hg; KO 101±5 mm Hg); hemodynamic data were obtained in n=3 WT mice and n=5 KO mice. As shown in Figure 5A, Gdf-15–deficient mice did not express GDF-15 in the myocardium. The size of the area-at-risk during coronary occlusion was comparable in WT and Gdf-15–deficient mice; however, myocardial infarct sizes after reperfusion were significantly larger in Gdf-15–deficient mice (Figure 5B and 5C). Notably, virtually identical results were obtained in male and female mice (supplemental Figure I). Greater infarct sizes in Gdf-15–deficient mice were associated with an enhanced occurrence of TUNEL− cardiomyocytes in the infarct border zone (Figure 5D and 5E). Under baseline conditions, the rate of TUNEL− cardiomyocytes was very low (<0.1%) in Gdf-15–deficient and WT mice (n=2 each).

GDF-15 Protects Cultured Cardiomyocytes During Simulated Ischemia and I/R

LDH release, a biochemical marker of necrotic cell death, was significantly increased after 3 hours of simulated ischemia (Figure 6A). The same treatment did not increase
apoptotic cell death in our culture model, as indicated by FACS analysis (number of annexin V<sup>pos</sup>/PI<sup>neg</sup> cells), histone ELISA, and TUNEL/Hoechst staining (data not shown). However, simulated ischemia for 3 hours followed by reperfusion for 1 hour strongly induced cardiomyocyte apoptosis, as shown by these three assays (Figure 6B through 6F). In contrast, no further LDH release was detected, indicating that necrosis does not contribute significantly to cell death during reperfusion (data not shown). Pretreatment of cardiomyocytes with recombinant GDF-15 diminished LDH release during a subsequent 3-hour episode of simulated ischemia (Figure 6A). Similarly, GDF-15 reduced the number of annexin V<sup>pos</sup>/PI<sup>neg</sup> cells (Figure 6B and 6C), the formation of histone-associated DNA fragments (Figure 3D), and the number of TUNEL<sup>pos</sup> cells after 3 hours of simulated ischemia followed by 1 hour of reperfusion (Figure 6E and 6F). The cytoprotective effects of GDF-15 were comparable to the effects of LIF (Figure 6A and 6D), an IL-6–related cytokine that has previously been shown to protect from I/R injury.22

**GDF-15 Protects Cardiomyocytes Via PI3K- and Akt-Dependent Mechanisms**

A number of growth factors and cytokines protect the heart from I/R injury via the PI3K–Akt signaling pathway.23 We therefore explored whether this pathway is important for the cardioprotective effects of GDF-15. As shown in Figure 6, the cytoprotective effects of GDF-15 during simulated ischemia or I/R were abolished by the PI3K inhibitors LY294002 and wortmannin, indicating that PI3K is required for the prosurvival effects of GDF-15.

GDF-15 promoted a rapid and transient Ser<sup>473</sup> phosphorylation (activation) of Akt in cardiomyocytes (Figure 7A). Enhanced Ser<sup>473</sup> phosphorylation of Akt was paralleled by an increase in Ser<sup>136</sup> phosphorylation (inactivation) of the Akt downstream target Bad, a Bcl family member known to inhibit Bcl survival proteins (Figure 7B). To assess whether Akt activation is required for the protective effects of GDF-15, cardiomyocytes were infected with a replication-deficient adenovirus encoding a dominant-negative, kinase-inactive mutant of Akt1. Adenoviral expression of dnAkt1 abolished the protective effects of GDF-15 during a subsequent episode of simulated I/R as assessed by TUNEL/Hoechst staining (Figure 7C and 7D) and histone ELISA (Figure 7E). Infection with a control virus had no effects (Figure 7C through 6E).

**Discussion**

The present study identifies the TGF-β superfamily member GDF-15 as a cytokine that is strongly induced in the myocardium after ischemic injury, and that provides endogenous protection against I/R-induced cardiomyocyte apoptosis, possibly via PI3K–Akt-dependent signaling pathways. Together with the report by Xu et al,21 our study is the first to demonstrate that GDF-15 has a functional role in the cardiovascular system and, in fact, the first study that assigns an in vivo function to GDF-15.

Two mouse models were used in our study (permanent coronary artery ligation and transient ligation followed by reperfusion) to simulate the distinct clinical scenarios in patients with AMI not receiving or receiving reperfusion therapy. Permanent ischemia and transient ischemia followed by reperfusion both led to a robust induction of GDF-15–deficient mice develop greater infarcts sizes after I/R. Gdf-15 gene-targeted mice (KO) and their WT littermates underwent transient LAD ligation for 1 hour followed by reperfusion for 24 hours. GDF-15 and α-actinin expression levels were determined by immunoblotting in the area-at-risk (AAR) in WT and Gdf-15 KO mice (A). AAR and infarct sizes were determined by Evans blue and TTC staining. Representative cross-sections are shown (B). The area of the myocardium not stained with Evans blue represents the AAR, infarcted areas appear pale (highlighted with red dots), and viable myocardium appears pink (highlighted with green stripes). Data from n=17 Gdf-15 KO mice and n=20 WT littermates are summarized (C). The AAR and infarcted area (MI) were expressed as percentage of LV cross-sectional area (LV); MI was also calculated as percentage of AAR. Apoptotic cardiomyocytes in the infarct border zone were detected by TUNEL/Hoechst staining and anti–α-actinin immunostaining. Typical sections are shown (D): TUNEL (left panels), Hoechst (middle), TUNEL/Hoechst/α-actinin overlay (right). Data from n=7 WT and n=8 Gdf-15 KO animals are shown (E). *P<0.05 vs WT.
GDF-15 mRNA and pro-peptide expression levels in the myocardium at risk. GDF-15 expression in the remote myocardium was induced only transiently and to a lesser extent, suggesting that the ischemic insult per se rather than early neurohormonal activation or increases in ventricular wall stress promotes GDF-15 expression in the injured myocardium. Emphasizing the potential clinical relevance of our findings, GDF-15 pro-peptide levels were also upregulated in autopsy samples obtained from patients with a fatal AMI (regardless of reperfusion therapy). At the cellular level, cardiomyocytes within irreversibly damaged myocardial areas robustly expressed GDF-15 in AMI patients.

Supporting the conclusion that cardiomyocytes are a major source of GDF-15 expression after ischemic injury, isolated rat cardiomyocytes, when exposed to simulated ischemia or I/R, strongly expressed GDF-15. Inducible NOS2, which may contribute to I/R injury by enhancing nitrosative stress, appeared to be involved in the upregulation of GDF-15 in cardiomyocytes after I/R via NO–peroxynitrite-dependent signaling pathways. Along this line, IL-1β/IFN-γ enhanced GDF-15 expression in cardiomyocytes via induction of endogenous NOS2 and nitrosative stress. Notably, GDF-15 expression in tumor cell lines has been shown to involve the redox-sensitive transcription factors p53 and Egr-1, both of which are also activated by I/R in cardiomyocytes. Because previous studies have shown that GDF-15 is upregulated in cortical neurons after cryoinjury and in hepatocytes after toxic liver injury, it appears that induction of GDF-15 might be a generic response to external stressors.

We detected mostly the pro-peptide of GDF-15 in cardiomyocyte extracts and in cardiac tissue samples, suggesting that the mature peptide is efficiently secreted. Indeed, conditioned supernatants obtained from cardiomyocytes treated with NO or subjected to simulated ischemia or I/R contained predominantly the mature GDF-15 peptide. This is in good agreement with data obtained in cultured human kidney cells showing that mature GDF-15 is not stored but rapidly secreted.

The phenotype of Gdf-15 gene-targeted mice underscores the in vivo functional significance of these findings. Gdf-15–deficient mice have virtually normal hearts under nonstressed, baseline conditions. Moreover, baseline heart rate, blood pressure, and maximal LV pressure were comparable in Gdf-15–deficient and WT mice. However, Gdf-15–deficient mice developed greater infarct sizes and more cardiomyocyte apoptosis in the infarct border zone.
after I/R injury compared with WT littermates, indicating that endogenous GDF-15 limits myocardial tissue damage in vivo. To gain mechanistic insight into the cardioprotective effects of GDF-15, we set up a cell culture model of simulated I/R. Consistent with previous investigations, simulated ischemia was related to an increased rate of necrosis, whereas reperfusion led to accelerated apoptosis in our cell culture model. However, it should be pointed out that the mode of cell death contributing to I/R injury depends, to some extent, on experimental conditions, and that necrosis and apoptosis represent only the extremes of a continuum of various modes of cell death. Using four complementary techniques to assess cell death during simulated I/R, our data indicate that GDF-15, when added 1 hour before the ischemic event, suppresses necrosis during ischemia and apoptosis during subsequent reperfusion. GDF-15 was used at a concentration of 20 ng/mL during these cell culture studies, a dose that is probably pathophysiologically relevant: normal human sera contain 0.5 ng/mL of GDF-15. However, up to 30-fold higher GDF-15 serum levels have been reported in specific situations (eg, during pregnancy or in patients with certain types of cancer). Preliminary data from our laboratory, obtained with a recently established immunoradiometric assay, indicate that GDF-15 serum levels increase up to 6 to 10 ng/mL in AMI patients presenting for primary angioplasty. Given that the infarcted human myocardium appears to be a major site of GDF-15 production, even higher concentrations are probably achieved at the tissue level.

The pro-survival effects of GDF-15 in cultured cardiomyocytes subjected to simulated I/R were associated with a rapid activation of the serine–threonine kinase Akt. More important, from a mechanistic standpoint, the protective effects of GDF-15 were abolished by PI3K inhibitors and by adenoviral expression of a dominant-negative Akt1 mutant, pointing toward a critical involvement of the PI3K–Akt signaling pathway in the cytoprotective effects of GDF-15. It is noteworthy in this regard that GDF-15 also promotes Akt activation in cardiomyocytes when added at the time of reperfusion (data not shown), suggesting that GDF-15 may have therapeutic potential for the treatment of myocardial I/R-injury, a hypothesis that should be tested in future studies.

Our data indicate that Bad may be a target of GDF-15–activated Akt in cardiomyocytes; however, the precise downstream effectors mediating the prosurvival effects of GDF-15 remain to be established.
in this context that PI3K can protect the heart from I/R injury also via Akt-independent pathways.32 Moreover, PI3K–Akt-independent pathways might be involved in the protective effects of GDF-15; as shown Xu et al, GDF-15 activates extracellular signal-regulated kinases in cultured cardiomyocytes,21 which have been suggested to exert antiapoptotic effects in the myocardium.33 In any case, our observations are consistent with the concept that distinct cell survival signals converge at the PI3K–Akt signaling pathway in cardiomyocytes.13,34–37 Finally, considering observations are consistent with the concept that distinct in vitro via PI3K–Akt,8,27 our data lend support to the notion that GDF-15 expression is induced in lesioned cortical myocyte hypertrophy.38,39

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References


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MATERIALS AND METHODS

Materials. Human GDF-15, rat leukemia inhibitory factor (LIF), interleukin (IL)-1β, and interferon (IFN)-γ were purchased from R&D Systems, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazide (AMT), N(G)-nitro-l-arginine methyl-ester (L-NAME), and tetramethylammonium-peroxynitrite from Alexis, Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP) from Calbiochem, and 8-para-chlorophenylthio-cGMP (8-pCPT-cGMP) from Biolog.

Cardiomyocyte culture and recombinant adenoviruses. Ventricular cardiomyocytes were isolated from 1- to 3-day-old Sprague-Dawley rats.1 Cells were plated in gelatin-coated culture dishes in DMEM/medium 199 (4:1), supplemented with 10% horse serum, 5% FCS, glutamine, and antibiotics. The next morning, cells were switched to DMEM/medium 199 supplemented only with glutamine and antibiotics (maintenance medium). The replication-deficient adenovirus encoding dominant-negative Akt1 (K179M mutation, Ad.dnAkt1) was kindly provided by Dr. Richard Patten (Tufts University).2 Cells were infected for 2h in maintenance medium (MOI=50), washed and kept in maintenance medium supplemented with 5% FCS for 24h, and finally switched to maintenance medium without serum.

Northern blot and real-time PCR. Total RNA was isolated by the TriFast reagent (PeqLab). GDF-15 mRNA expression in cultured cardiomyocytes was determined by Northern blot using a 520 bp rat GDF-15 cDNA probe generated by PCR cloning (forward: TGCTGAGCCGACTGCATGC, reverse: CATGCTCAGTTGCAGCTGAC).3 GDF-15 mRNA expression in mouse hearts was determined by real-time PCR. Following reverse transcription (Superscript II, Invitrogen), real-time PCR was performed using the Brilliant SYBR Green Mastermix-Kit and the MX4000 multiplex QPCR System from Stratagene (1 min denaturation at 95°C, 1 min annealing at 57°C, 1 min elongation at 72°C; 40 cycles). PCR primers were designed based on the murine GDF-15 cDNA sequence (forward: ACGAGCTACGGGCTCGGC, reverse: CCCAATCTCCTCTGGACTG).4 Data were acquired at 80°C to avoid measurement of non-specific products. PCR-efficiency was >95% as revealed by standard curve slope calculation. Melting curve analysis showed no non-specific amplification products or primer dimers. GDF-15 mRNA expression was normalized against 18S rRNA or GAPDH mRNA expression quantified by Northern blot or real-time PCR, respectively.
Immunoblotting. GDF-15 protein levels in rat cardiomyocytes and culture supernatants, and in mouse hearts were quantified by immunoblotting using a polyclonal antibody raised against a peptide sequence (HRTDSGVSLQTYDDL) from the C-terminus of rat GDF-15; this antibody recognizes both the pro-peptide and the secreted peptide of rat and mouse GDF-15, but could not be used to detect GDF-15 in human myocardium (generation of this antibody is described in Ref. 3). Culture supernatants were concentrated by Amicon Ultra-4 MWCO 5000 filter devices (Millipore) prior to immunoblotting. A polyclonal goat anti-human GDF-15 IgG antibody from R&D Systems (Catalog No. AF957) was used to determine GDF-15 expression levels in human myocardium (this antibody could not be used to detect rat or mouse GDF-15). Antibodies against α-actinin, Akt, Ser473-phospho-Akt, Bad, and Ser136-phospho-Bad expression levels were obtained from Sigma (α-actinin) or New England Biolabs (all other).

Simulated ischemia and I/R in vitro. Cardiomyocytes were exposed to simulated ischemia or I/R as described. In brief, cells were switched from maintenance medium to a buffer containing (in mmol/L) 137 NaCl, 12 KCl, 0.5 MgCl2, 0.9 CaCl2, 4 HEPES, 10 2-deoxy-glucose, and 20 sodium-lactate (pH 6.2), and were incubated at 37°C in a hypoxia chamber (Modular Incubator Chamber-101, Billups-Rothenberg) flushed with 5% CO2 and 95% N2 (simulated ischemia). Control cells were cultured in a buffer containing (in mmol/L) 137 NaCl, 3.8 KCl, 0.5 MgCl2, 0.9 CaCl2, 4 HEPES, 10 glucose, and 20 pyruvate (pH 7.4), and incubated at 37°C in an atmosphere containing 5% CO2 and 95% room air. After various time intervals, cells were switched back to maintenance medium and kept in 5% CO2 and 95% room air at 37°C (simulated reperfusion).

Assessment of cell death after simulated ischemia and I/R in vitro. Lactate dehydrogenase (LDH) release was measured by a cytotoxicity detection kit (Roche). Formation of histone-associated DNA fragments was quantified by Cell Death Detection ELISA (Roche). DNA fragmentation was determined by in situ TdT-mediated dUTP nick end-labeling (TUNEL) using the ApopTag fluorescein apoptosis detection kit (Serologicals Corporation). After nuclear counter-staining with Hoechst 33258, the number of TUNELpos nuclei with condensed nuclear chromatin was determined by fluorescence microscopy and expressed as the percentage of all Hoechstpos nuclei. Moreover, cardiomyocytes were labeled with annexin V and propidium iodide (PI) and analyzed by flow cytometry (FACSCalibur, BD Biosciences).
**Surgical procedures.** All animal procedures were approved by our local state authorities. Myocardial GDF-15 expression levels were determined in 8 to 12-week-old male C57BL/6 mice subjected to permanent or transient left anterior descending coronary artery (LAD) ligation, as described previously. In brief, mice were anesthetized and ventilated with isoflurane (1-2%). A left thoracotomy was performed, and the LAD was ligated with a slipknot, which was left in place (permanent ischemia), or removed 1h later (I/R). Control mice underwent a sham operation. At various time points, mice were sacrificed, left ventricles (LVs) were removed, divided into the injured area (anteroapical wall, distal to the ligation site) and remote myocardium (basal part of the interventricular septum), and snap-frozen in liquid N2. Gdf-15 gene-targeted mice were kindly provided by Dr. Se-Jin Lee (Johns Hopkins University) and maintained on a C57BL/6/129/SvJ background. Eight to 16-week-old male and female Gdf-15 deficient mice and their wild-type littermates were subjected to transient LAD ligation. Area-at-risk and infarct sizes were determined by Evans blue and TTC staining and computerized planimetry. Apoptotic cardiomyocytes in the infarct border zone were detected by TUNEL and Hoechst staining (cardiomyocytes were identified by anti-α-actinin immunostaining). Heart rate, mean arterial blood pressure, and maximal left ventricular pressure were determined in a closed chest preparation using a 1.4 F Millar catheter advanced via the right carotid artery into the ascending aorta and LV, as described previously.

**Human myocardial tissue.** Ethical approval was obtained from the Ethics Committee at the University Medical Center in Amsterdam. LV myocardial tissue samples were collected at autopsy from 17 patients who had died from an AMI (10 males, mean age 66±3 years) and from 5 patients who had died from non-cardiac causes (2 males, mean age 59±9 years). Autopsy was performed within 24h after death. Tissue samples were taken from the infarcted myocardium (delineated by decreased LDH staining), and stored in liquid N2.

**Immunohistochemistry.** The expression pattern of GDF-15 in human myocardial tissue samples was determined by immunohistochemistry using the anti-human GDF-15 antibody from R&D Systems (1:250 dilution), and a secondary horseradish peroxidase-conjugated rabbit anti-goat antibody from Dakopatts (1:25 dilution). Viable and irreversibly damaged (jeopardized) myocardial areas were discriminated by anti-complement C3d staining using the C3-15 monoclonal antibody, as described. Slides were finally washed and incubated in PBS...
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containing 3,3’-diamine-benzedrine-tetrahydrochloride (0.5 mg/mL) and 0.01% H₂O₂.

Statistical analysis. Data are presented as means±SEM. The number of experiments refers to the number of mice, patients, or independent cardiomyocyte preparations. Differences between groups were analyzed by one-way ANOVA followed by Student-Newman-Keuls post hoc test. A two-tailed P value <0.05 was considered to indicate statistical significance.

REFERENCES


Area-at-risk and infarct sizes after ischemia/reperfusion in male and female wild-type and Gdf-15 gene-targeted mice. Gdf-15 gene-targeted mice (KO) and their wild-type (WT) littermates underwent transient left anterior descending coronary artery ligation for 1h followed by reperfusion for 24h. The area-at-risk (AAR) and infarcted area (MI) were determined by Evan’s blue and TTC staining. The AAR and infarcted area (MI) were expressed as [%] of left ventricular cross-sectional area (LV); MI was also calculated as [%] of AAR. Data from 8 male and 9 female KO mice and 12 male and 8 female WT mice are presented.