UNIVERSITÀ DEGLI STUDI FIRENZE

FLORE
Repository istituzionale dell'Università degli Studi di Firenze

Hyperglycemia activates JAK2 signaling pathway in human failing myocytes via angiotensin II-mediated oxidative stress.

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

Hyperglycemia activates JAK2 signaling pathway in human failing myocytes via angiotensin II-mediated oxidative stress / MODESTI A.; BERTOLOZZI I.; GAMBERI T.; MARCHETTA M.; LUMACHI C.; COPPO M.; MORONI F.; TOSCANO T.; LUCCHESE G.; GENSINI G.F.; PA. MODESTI. - In: DIABETES. - ISSN 0012-1797. - ELETTRONICO. - 54:(2005), pp. 394-401. [10.2337/diabetes.54.2.394]

Availability:

This version is available at: 2158/311937 since: 2016-01-13T12:49:06Z

Published version:

DOI: 10.2337/diabetes.54.2.394

Terms of use:

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf)

Publisher copyright claim:

(Article begins on next page)

12 October 2024

Hyperglycemia Activates JAK2 Signaling Pathway in Human Failing Myocytes via Angiotensin II–Mediated Oxidative Stress

Alessandra Modesti,[1] Iacopo Bertolozzi,[2] Tania Gamberi,[1] Matilde Marchetta,[2] Camilla Lumachi,[2] Mirella Coppo,[2] Federico Moroni,[2] Thomas Toscano,[3] Gianluca Lucchese,[3] Gian F. Gensini,[2] and Pietro A. Modesti[2]

Hyperglycemia was reported to enhance angiotensin (Ang) II generation in rat cardiomyocytes, and Ang II inhibition reduces cardiovascular morbidity and mortality in diabetic patients. In diabetic patients, the enhanced activation of intracellular pathways related with myocyte hypertrophy and gene expression might enhance the progression of cardiac damage. Therefore, we investigated the effects of glucose on Ang II–mediated activation of Janus-activated kinase (JAK)-2, a tyrosine kinase related with myocyte hypertrophy and cytokine and fibrogenetic growth factor overexpression, in ventricular myocytes isolated from nonfailing human hearts (n = 5) and failing human hearts (n = 8). In nonfailing myocytes, JAK2 phosphorylation was enhanced by Ang II only in the presence of high glucose (25 mmol/l) via Ang II type I (AT1) receptors (+79% vs. normal glucose, $P < 0.05$). JAK2 activation was prevented by inhibitors of reactive oxygen species (ROS) generation (diphenyleneiodonium [DPI], tiron, and apocynin). In myocytes isolated from failing hearts, JAK2 phosphorylation was enhanced by high glucose alone (+107%, $P < 0.05$). High glucose–induced JAK2 activation was blunted by both ACE inhibition (100 nmol/l ramipril) and AT1 antagonism (1 μmol/l valsartan), thus revealing that the effects are mediated by autocrine Ang II production. Inhibition of ROS generation also prevented high glucose–induced JAK2 phosphorylation. In conclusion, in human nonfailing myocytes, high glucose allows Ang II to activate JAK2 signaling, whereas in failing myocytes, hyperglycemia alone is able to induce Ang II generation, which in turn activates JAK2 via enhanced oxidative stress. *Diabetes* 54: 394–401, 2005

From the [1]Department of Scienze Biochimiche, University of Florence, Florence, Italy; [2]Clinica Medica e Cardiologia, University of Florence, Florence, Italy; and the [3]Institute of Thoracic and Cardiovascular Surgery, University of Siena, Siena, Italy.

Address correspondence and reprint requests to Prof. Pietro Amedeo Modesti, MD, PhD, Clinica Medica e Cardiologia, University of Florence, Viale Morgagni 85, 50134 Florence, Italy. E-mail: pamodesti@unifi.it.

Received for publication 4 April 2004 and accepted in revised form 11 November 2004.

AGTN, angiotensinogen; Ang, angiotensin; AT1, Ang II type I; AT2, Ang II type II; DMEM, Dulbecco's minimal essential medium; DPI, diphenyleneiodonium; ERk, extracellular signal–related kinase; JAK, Janus-activated kinase; MAPK, mitogen-activated protein kinase; RAS, renin-angiotensin system; ROS, reactive oxygen species.

© 2005 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

In patients with diabetes and abnormal glucose tolerance, left ventricular hypertrophy is often detectable (1,2) and diastolic dysfunction is highly prevalent (3). Diabetic cardiomyopathy, characterized by both myocyte hypertrophy and enhanced collagen deposition (4), is almost invariably followed by the transition into a dilated form with depressed contractile behavior and heart failure irrespective of coronary heart disease and hypertension (4,5).

A direct link was found among glucose, angiotensin (Ang) II, reactive oxygen species (ROS) generation, and the transmission of death signaling in rat myocytes (p38 mitogen-activated protein kinase [MAPK]). Indeed, the glycosylation of *O*-linked *N*-acetylglucosamine may activate p53 in cardiomyocytes, thus enhancing angiotensinogen (AGTN) transcription and Ang II formation (6), which is finally responsible for the activation of death-related intracellular signaling pathways (4,6). In these experimental studies, hyperglycemia-induced Ang II formation failed to activate extracellular signal–related kinase (ERK)-1/2 (6), a MAPK playing a well-accepted role in the hypertrophic response of cardiomyocytes (7,8), so that the possible relationship between glucose and Ang II in diabetic myocyte hypertrophy remains elusive.

In other cell types, the signaling cascade linking the activation of the nuclear transcriptional changes and cell growth to Ang II type I (ATI) receptor stimulation was found to include the phosphorylation of Janus-activated kinase (JAK)-2, a soluble tyrosine kinase (9) also involved in the transmission of the inflammatory processes (10). Ang II–mediated JAK2 activation in vitro was also found to critically require ROS generation via the membrane-bound NADPH oxidase system (10). However, the capability of Ang II to induce JAK2 phosphorylation in cardiomyocytes was observed only in neonatal rat cells (11–13), whereas no information is available in adult ventricular myocytes.

In rat mesangial cells, Ang II–mediated JAK2 activation in vitro was found to be enhanced in the presence of high glucose concentration (14), and high glucose is known to enhance both ROS generation (15) and Ang II formation (6). However, although there have been several studies showing that high glucose potentiates local renin-angiotensin system (RAS) in cardiomyocytes of experimental animals, no study was conducted in human ventricular

TABLE 1
Characteristics of subjects investigated

	Nonfailing	Dilated cardiomyopathy
Age (years)	47 ± 5	53 ± 11
Sex (M/F)	4/1	7/1
Body surface area (m^2)	1.81 ± 0.13	1.83 ± 0.26
New York Heart Association class (III/IV)	—	6/2
Left ventricular end diastolic diameter index (mm/m^2)	26.1 ± 3.6	44.1 ± 6.7*
Left ventricular mass index (g/m^2)	101 ± 16	266 ± 49*
Ejection fraction (%)	63 ± 3.2	19 ± 7.5*
Cardiac index ($l \cdot m^{-2} \cdot min^{-1}$)	—	2.03 ± 0.40
Mean pulmonary artery pressure (mmHg)	—	22.7 ± 8.6
Left ventricular end diastolic pressure (mmHg)	—	19.4 ± 7.3
Pulmonary capillary wedge pressure (mmHg)	—	14 ± 4
End systolic stress ($kdyne/cm^2$)	—	99 ± 18
End diastolic stress ($kdyne/cm^2$)	—	22 ± 5

Data are means ± SE. *$P < 0.01$ vs. control subjects.

myocytes, and the mechanism for activated local RAS in that condition remains elusive. It has been shown that chymase rather than ACE is a major determinant for local Ang II generation in the canine (16), baboon (17), and human (18) hearts. These observations tend to minimize the role of ACE in the conversion of Ang I to Ang II in human heart failure. However, opposite results have also been reported (19), and ACE, rather than chymase, was found to be overexpressed in human failing hearts (20). One of the problems with these findings is that little attention has been given to myocytes even though the entire myocardium or the properties of the interstitial fluid have been analyzed (16–20).

In this article, the hypothesis was raised that glucose and Ang II might interact to enhance NADPH oxidase activation and JAK2 phosphorylation in human ventricular myocytes. In particular, we investigated whether high glucose may enhance JAK2 activation in human myocytes, the role played by Ang II, and the pathways involved in high glucose–enhanced Ang II formation in failing myocytes.

RESEARCH DESIGN AND METHODS

Myocytes were obtained from the hearts of five putative organ donors (aged 47 ± 5 years) with no histories or signs of heart disease and whose hearts could not be transplanted because of noncardiac reasons ("nonfailing hearts") and of eight patients (aged 53 ± 11 years) with end-stage dilated cardiomyopathy scheduled to undergo cardiac transplantation. Characteristics of subjects investigated are reported in Table 1.

Subjects with arterial hypertension, history of myocardial infarction, and echocardiographic evidence of valve or congenital heart disease were not considered for the study. Diagnosis of dilated cardiomyopathy was based on clinical and echocardiographic examination. Myocardial tissue was obtained from a portion of the left ventricular free wall during cardiac transplantation.

The protocol of this study complies with the principles of the Helsinki Declaration and was approved by our institution (University of Florence). All patients gave their informed written consent to participate and to have their hearts used for the study.

Myocyte isolation. Myocytes were isolated with the enzymatic digestion method as previously described (20). Briefly, a coronary artery branch was cannulated and perfused for 10 min with a calcium-free buffer (blood washout) and then with collagenase (Worthington-type II, 100 units/ml, 20 ml/min). The collagenase-perfused tissue was then minced and shaken in a resuspension buffer (HEPES-MEM buffer supplemented with bovine serum albumin 0.5%, 0.3 mmol/l $CaCl_2$, and 10 mmol/l taurine). Myocytes were then collected and enriched by centrifugation for 4 min at 35*g*. Cells were then suspended in isotonic Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) at a final concentration of 41% and centrifuged for 10 min at 34*g*. Pellets were washed and smears were made (Fig. 1). Rod-shaped, trypan blue–excluding cells constituted nearly 80% of all myocytes. Nonmyocytes accounted for <2% of the cells (20).

Receptor binding studies. Equilibrium binding studies were performed by incubating freshly isolated myocytes (1×10^6 cell/ml) with 100 pmol/l of [^{125}I]Ang II (2,000 Ci/mmol; Amersham Biosciences, Uppsala, Sweden) and increasing concentrations of unlabeled Ang II (0–100 μmol/l) at 22°C for 90 min as previously described (20). Ang II receptor subtypes were characterized

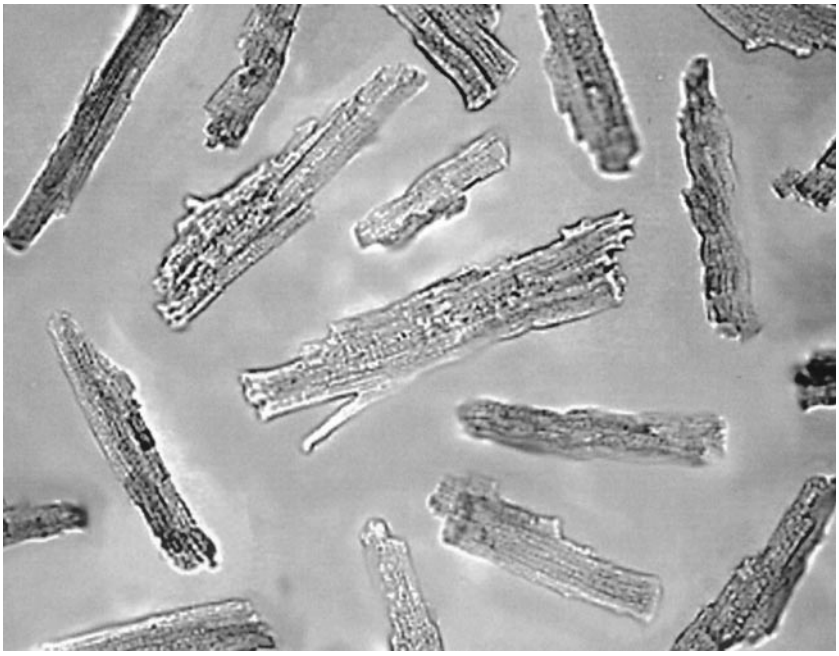

FIG. 1. Myocytes from the left ventricle of a patient with dilated cardiomyopathy.

using selective antagonists for AT1 (valsartan, a kind gift of Novartis, Italy) and Ang II type II (AT2) receptors (PD123319). Binding data were analyzed by a nonlinear fitting computer program (LIGAND).

RT-PCR. To document the presence of transcripts for the two Ang receptor subtypes, for other local RAS components, and for chymase in human ventricular nonfailing and failing myocytes, total mRNA was extracted with TRIzol reagent (BRL-Life Technologies), as indicated by the manufacturer, and reverse-transcribed using oligo dT as previously reported (20). The resulting cDNA was amplified using specific primers for AGTN (20), ACE (21), AT1 receptor (22), AT2 receptor (22), chymase (18), and GAPDH used as internal standard (20). To ensure that different amounts of PCRs on myocardial biopsies were not due to markedly different mRNA starting concentrations, PCR analysis for GAPDH was performed on serial twofold dilutions of cDNA for each sample. The last dilution giving a positive reaction for GAPDH was used to equalize the amount of cDNA used in each PCR.

PCRs were performed in a DNA Thermal Cycler (Perkin Elmer Cetus), band densities were analyzed using a computer image densitometer (Qwin; Leica), and the densitometric ratio to GAPDH was calculated for each transcript (20).

Incubation of myocytes with high glucose or Ang II. Freshly isolated myocytes (10^5 cell/ml) from both nonfailing and dilated cardiomyopathy hearts were suspended in Dulbecco's minimal essential medium (DMEM) (pH 7.4) and in the absence of insulin. After 30 min, the medium was changed with DMEM containing 5.5 mmol/l (normal glucose) or 25 mmol/l glucose (high glucose) corresponding to plasma levels of 100 and 450 mg/dl, respectively. Hyperosmolarity was corrected by decreasing salts (final osmolarity 322 mOsm/kg), and acidosis was corrected by adding 20 mmol/l HEPES. In separate experiments, the effect of osmolarity was checked by adding 19.5 mmol/l of mannitol or glucose to normal glucose buffer. The medium was supplemented with 100 units/ml penicillin G and 0.1 mg/ml streptomycin. The cells were incubated under an atmosphere of 95% air plus 5% CO_2 at 37°C for 2, 6, and 12 h.

To investigate the relative role of Ang II receptor subtypes and the involvement of the RAS in high glucose–induced JAK2 activation, myocytes were preincubated with selective AT1 (1 μmol/l valsartan) or AT2 (1 μmol/l PD123319) receptor antagonists and with an ACE inhibitor (100 nmol/l ramipril).

In addition, to try to characterize ROS induced by Ang II, we performed experiments in the presence of a cell-permeable superoxide anion scavenger (100 μmol/l tiron), a flavoprotein inhibitor (100 μmol/l diphenyleneiodonium [DPI]), an inhibitor of NADPH oxidase system (10 μmol/l apocynin), and an inhibitor of mitochondrial ROS production (5 μmol/l rotenone).

These compounds were added 30 min before glucose. Preliminary experiments revealed that the incubation of myocytes with tiron, diphenyleneiodonium (DPI), apocynin, and rotenone in the absence of Ang II and in normal glucose did not cause any nonspecific effect on JAK2 phosphorylation status.

To investigate whether glucose may affect Ang II signaling, myocytes were then coincubated with Ang II (100 nmol/l) in the presence of AT1 or AT2 receptor antagonists, tiron, DPI, apocynin, and rotenone at the concentrations reported above, with normal glucose or high glucose. Myocytes were then separated and frozen at −80°C.

Western blot. Myocytes were then suspended in ice-cold lysis buffer (10 nmol/l Tris-HCl, 20 mmol/l NaCl, 1 mmol/l sodium orthovanadate, 10 mmol/l sodium pyrophosphate, 10 mmol/l okadaic acid, 2 mmol/l EGTA, 2 mmol/l dithiotreitol, and 1 mmol/l phenylmethylsulfonyl fluoride, pH 7.4). Lysates were kept on ice, sonicated four times for 5 s, and centrifuged for 10 min at 13,000*g*. Protein concentration in the supernatant fraction was assessed by the Bradford's method.

Samples (25 μg proteins) were then separated by 8% SDS-PAGE. The gel was then transferred to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences) and blocked by 1-h incubation at room temperature in TTBS (Tris-buffered saline with 0.05% Tween 20, pH 7.4) plus 5% skimmed milk powder. The nitrocellulose membrane was incubated overnight at 4°C with anti-human phospho-JAK2 (Upstate) or anti-human JAK2 antibodies (Santa Cruz). Then the nitrocellulose membrane was washed twice for 10 min with TTBS and incubated for 30 min with goat anti-rabbit IgG horseradish peroxidase conjugate antibody (Amerham Biosciences). After extensive washing, the bound antibody was visualized by a chemiluminescent detection system.

Positive control was performed by stimulating nonfailing myocytes with interleukin 6 (10 ng/ml). Negative controls were obtained both in the presence of competing peptide (Santa Cruz SC16566P) and with no first-antibody control lane.

Data analysis. Data are reported as means ± SD. Autoradiograms were analyzed by an image analyzer. All statistical comparisons were made using Student's *t* test for paired data and ANOVA. Significance was $P < 0.05$.

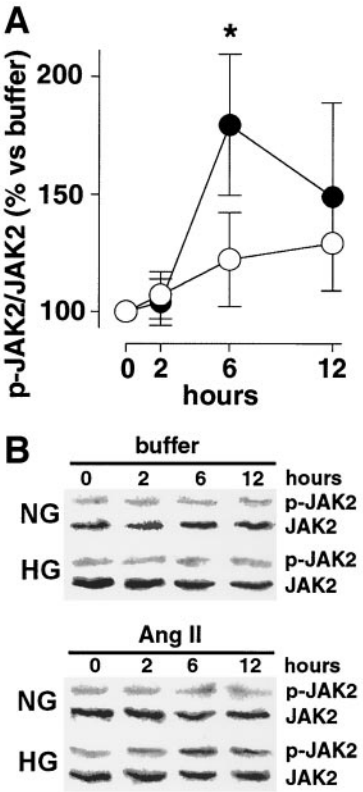

FIG. 2. A: Effect of Ang II (100 nmol/l) on the phosphorylated–to–total JAK2 ratio in the presence of normal (○) (5.5 mmol/l) or high glucose (●) (*$P < 0.01$ vs. normal glucose). In high glucose, osmolarity was kept normal (322 mOsm/kg) by reducing salt content. Data are means ± SD. *B*: Representative Western blots performed by using specific anti–human-phospho-JAK2 (*p*-JAK2) and anti-JAK2 (JAK2) antibodies on lysated myocytes at baseline and after 2, 6, and 12 h of incubation with normal or high glucose in the absence and in the presence of Ang II (100 nmol/l).

RESULTS

Effect of high glucose on Ang II–induced JAK2 activation in nonfailing myocytes. Incubation of myocytes isolated from nonfailing hearts in the presence of high glucose concentration (25 mmol/l) had no effect on JAK2 tyrosine phosphorylation (Fig. 2).

To investigate whether glucose may affect Ang II signaling, nonfailing myocytes were stimulated with Ang II in the presence of both normal glucose and high glucose. Ang II did not enhance JAK2 phosphorylation in nonfailing myocytes in the presence of normal glucose concentrations (NS vs. baseline) (Fig. 2). Conversely, when myocytes were preincubated in the presence of high glucose, Ang II caused a significant increase in JAK2 phosphorylation at 6-h (+79% vs. normal glucose, $P < 0.05$) concentrations (Fig. 2).

The lack of response observed in the presence of mannitol and the comparable effects of high glucose on JAK2 phosphorylation at normal and high final osmolarity (Fig. 3) indicate that the effect of glucose is independent of either reduction of salt content or osmolarity changes.

The effect is mediated by AT1 receptor as shown by the inhibitory effect of valsartan (1 μmol/l) (Fig. 4) on Ang II–induced JAK2 activation (61%, $P < 0.01$ for both). Conversely, AT2 antagonism did not cause any significant effect on Ang II–induced JAK2 phosphorylation (Fig. 4).

The coincubation with a flavoprotein inhibitor (DPI)

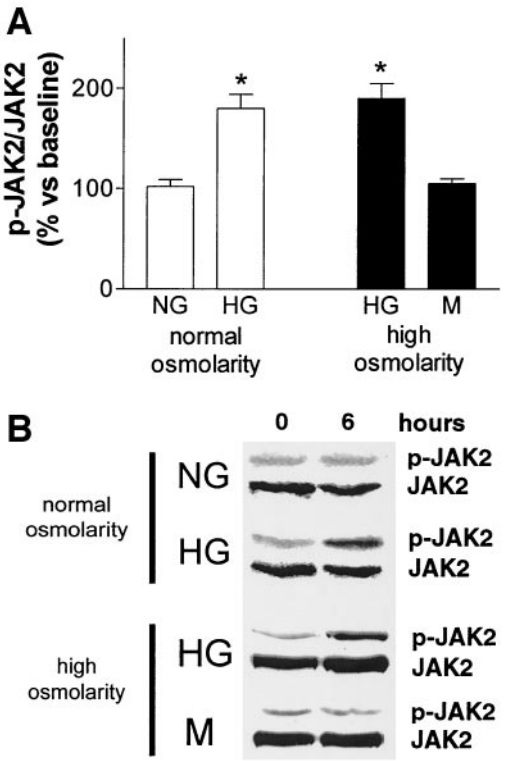

FIG. 3. *A*: Effects of 6-h incubation with Ang II (100 nmol/l) on the phosphorylated–to–total JAK2 ratio (*P < 0.01 vs. baseline). *B*: Representative Western blots performed with specific anti-JAK2 (JAK2) and anti–phospho-JAK2 (*p*-JAK2) antibodies on lysates of myocytes incubated for 6 h with Ang II (100 nmol/l) in DMEM containing either normal glucose (5.5 mmol/l) or high glucose (25 mmol/l) at the same final normal osmolarity. High osmolarity was obtained by adding 19.5 mmol/l of glucose (high glucose) or mannitol (M) to normal osmolarity normal glucose buffer.

prevented Ang II–induced JAK2 phosphorylation in high glucose conditions ($P < 0.05$ vs. Ang II) (Fig. 4), thus suggesting that JAK2 activation is mediated by ROS generation. Ang II–induced JAK2 phosphorylation in high glucose conditions was also inhibited by tiron, apocynin, and rotenone (Fig. 5).

Glucose induces JAK2 phosphorylation in failing myocytes. Differently from nonfailing myocytes, myocytes isolated from failing hearts responded to high glucose with a significant increase in the level of JAK2 tyrosine phosphorylation (207% vs. baseline, $P < 0.05$) (Fig. 6). Equal amounts of the nonphosphorylated form of JAK2 were detected in all samples (Fig. 6).

Ang II mediates the glucose-induced JAK2 phosphorylation in failing myocytes. Unlike that observed in nonfailing cells, Ang II induced JAK2 phosphorylation in failing myocytes also when the cells were incubated in the presence of normal glucose (Fig. 7).

To evaluate whether the high glucose–induced JAK2 phosphorylation was mediated by Ang II, myocytes were preincubated with AT1 (valsartan) or AT2 (PD123319) receptor antagonists. Valsartan significantly inhibited by 43% (Fig. 7) the high glucose–induced JAK2 phosphorylation in failing myocytes ($P < 0.01$ for both). Conversely, PD123319 did not affect glucose induced JAK2 phosphorylation (Fig. 7). The high glucose–induced JAK2 activation in failing myocytes was inhibited by 52% when the cells were preincubated with ramipril ($P < 0.01$ vs. buffer with high glucose) (Fig. 7), thus indicating that tyrosine phosphorylation was mediated by neo-formed Ang II. JAK2 activation was also prevented by preincubation with DPI (Fig. 7), tiron, apocynin, and rotenone.

The different response between failing and nonfailing myocytes was not due to different Ang II surface receptor populations because Ang II receptor binding on ventricular myocytes was not significantly different from nonfailing myocytes (Table 2). RT-PCR studies confirmed at the mRNA level the results obtained in binding studies for AT1 and AT2 receptor subtypes (Fig. 8). Conversely, a significant increase in both the AGTN and ACE mRNA ratios to GAPDH (4.4 ± 0.6–fold and 2.5 ± 0.4–fold vs. nonfailing cells, respectively) was found in failing cardiomyocytes.

DISCUSSION

The results of these experiments show that *1*) in nonfailing human ventricular myocytes Ang II activates JAK2 phosphorylation only in the presence of high glucose, which enhances ROS generation, whereas *2*) in failing myocytes, high glucose per se induces JAK2 phosphorylation via an ACE-dependent Ang II autocrine production.

High glucose enhances Ang II–induced JAK2 activation in nonfailing myocytes. Clinical studies have shown that in diabetic patients the inhibition of RAS may offer additional specific vascular and renal protection, beyond the lowering of blood pressure (23). A close link was reported to exist between high glucose, Ang II myocyte formation, and activation of intracellular pathway of death signaling (6). In particular, in rat cardiomyocytes high glucose induces enzymatic glycosylation of p53, which in turn activates Ang II production leading to p38 MAPK phosphorylation (6). In humans, diabetic cardiomyopathy is also characterized by myocyte hypertrophy (4). However, experimental studies in rats indicate that glucose induces only the phosphorylation of Jun NH_2-terminal kinase (JNK) (6), which plays a minor role in myocyte hypertrophy (24), whereas the MAPK pathway mainly related with myocyte hypertrophy (ERK1/2) (7,8) remained unaffected (6). No information was available regarding the response of a different intracellular pathway activated by JAK2 phosphorylation, which is known to play a relevant role in myocyte hypertrophy (25,26). Therefore, the observation that high glucose enhances Ang II–mediated JAK2 phosphorylation in nonfailing myocytes extend previous results revealing the possible involvement in diabetic cardiomyopathy of an intracellular pathway distinct from MAPK.

The second observation is that at variance with that reported in other cell types (10) and in neonatal myocytes (11–13), the stimulation of AT1 receptor subtype does not activate JAK2 in nonfailing human adult ventricular cardiomyocytes. The capability of the AT1 receptor to bind to the intracellular tyrosine kinase JAK2 was demonstrated and the AT1 receptor is known to contain a YIPP (tyrosine-isoleucine-proline-proline amino acids 319–322) motif in the cytoplasmic tail that may recruit JAK2 (27). However, although the biochemical structure creates a signaling complex mechanistically similar to that observed with growth factor and cytokine receptors, in our human adult myocytes, Ang II evoked a JAK2 response only in the presence of high glucose. Thus, a discrepancy exists

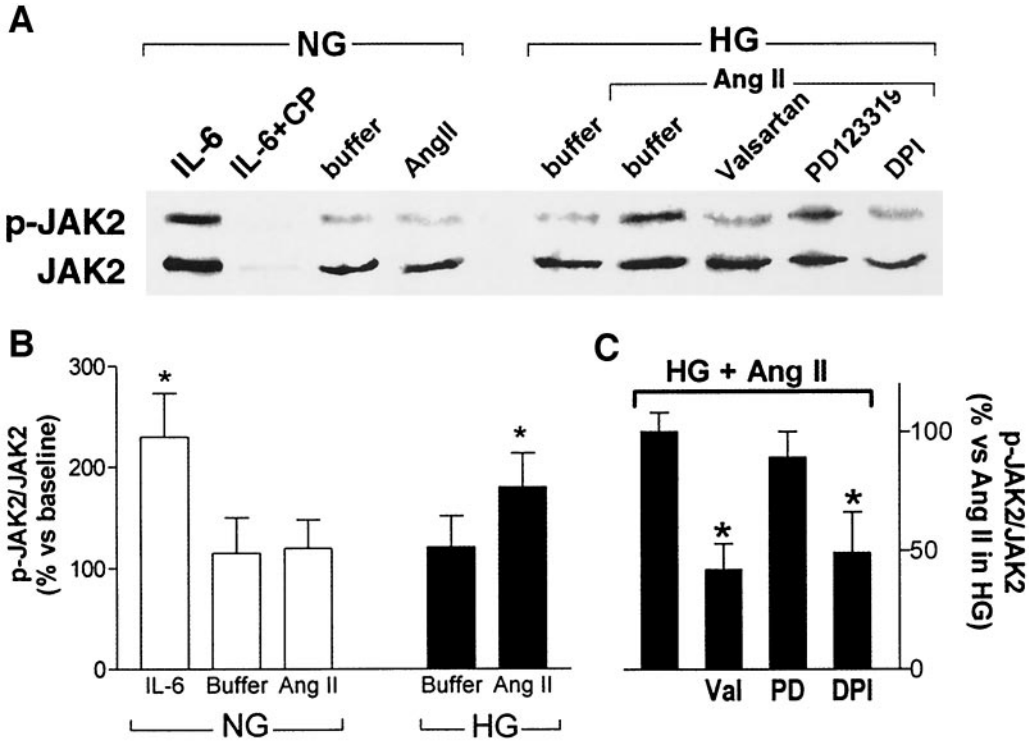

FIG. 4. Ang II–induced JAK2 phosphorylation in nonfailing human ventricular cardiomyocytes in the presence of high glucose concentrations is mediated by AT1 receptor subtype and ROS production. *A*: Representative Western blots obtained from nonfailing myocytes incubated in the presence of normal glucose or high glucose. Positive control was performed by stimulating nonfailing myocytes with interleukin-6 (10 ng/ml). Negative control was obtained in the presence of competing peptide (CP). Effects of AT1 (1 μmol/l valsartan) or AT2 (1 μmol/l PD123319) receptor antagonism and inhibition of ROS generation (100 μmol/l DPI). *B* and *C*: Phosphorylated–to–total JAK2 ratio quantitated from all experiments. Increases of *p*-JAK2–to–JAK2 ratio versus baseline (*B*) in normal glucose (□) and high glucose (■), and percent of inhibition of *p*-JAK2–to–JAK2 ratio versus Ang II in high glucose medium (*C*). Data are means ± SD.*$P < 0.01$.

between the response of neonatal cells and the close control over AT1-mediated JAK2 activation, which seems to operate in human adult ventricular myocytes. The different response might be related with the higher activity of intracellular phosphatase in adult than in neonatal cells (28). In the present study we did not measure the response of intracellular phosphatase to Ang II stimulation. However, phosphatase likely plays a minor role in Ang II–induced JAK2 response in the presence of high glucose because, although AT2 receptor subtype was reported to enhance phosphatase activity (29,30), the AT2 blockade failed to enhance JAK2 phosphorylation. A second possible relevant regulator of JAK2 response is represented by ROS. The capability of ROS to mediate JAK2 phosphorylation was observed in other cell types, such as fibroblasts and A-431 cells (31) and in aortic smooth muscle cells (10,32). In the present study the critical role of ROS also in human cardiomyocytes was demonstrated by the effects of tiron (a cell-permeable O_2 scavenger) and DPI (a nonspecific flavoprotein inhibitor). In the heart, NADPH oxidase is considered to be the main source of Ang II–induced O_2 production (10,33), but other possible sources of ROS in

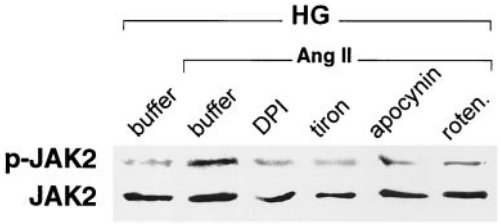

FIG. 5. Representative Western blots obtained from nonfailing myocytes incubated in high glucose and stimulated with Ang II in the presence of DPI (100 μmol/l), tiron (100 μmol/l), apocynin (10 μmol/l), and rotenone (roten.; 5 μmol/l).

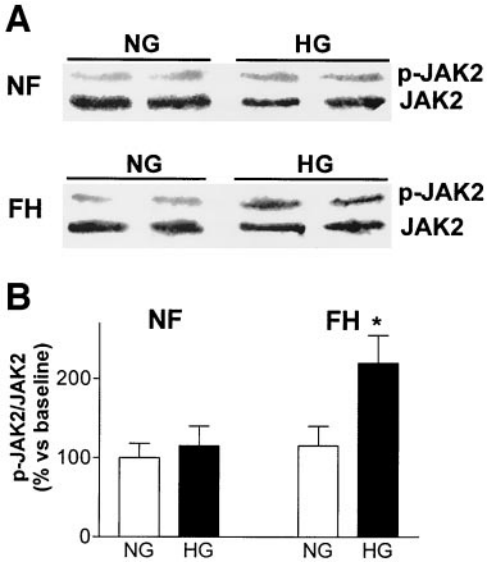

FIG. 6. *A*: Representative Western blots performed with anti–phospho-JAK2 (*p*-JAK2) and anti-JAK2 (JAK2) antibodies in myocytes obtained from two nonfailing (NF) and two failing hearts (FH) incubated for 6 h in the presence of normal glucose and high glucose. *B*: Increase in phosphorylated–to–total JAK2 ratio versus baseline in myocyte incubated in normal glucose (□) and high glucose (■). Data are means ± SD. *$P < 0.01$ vs. normal glucose.

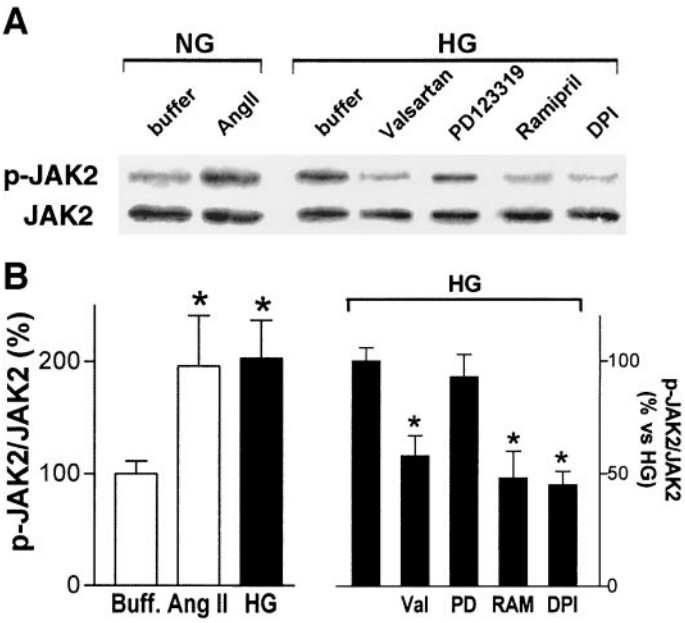

FIG. 7. *A*: High glucose–induced JAK2 phosphorylation in human ventricular myocytes isolated from failing hearts. *B*: Myocytes were incubated in normal glucose (□) or high glucose (■) in the presence of buffer or AT1 (1 μmol/l valsartan) and AT2 (1 μmol/l PD123319) receptor antagonists, ACE inhibitor (100 nmol/l ramipril), and inhibitor of ROS generation (100 μmol/l DPI).

diabetes are known to exist. In particular, in different cell types high glucose was reported to induce O_2 generation not only via NADPH oxidase but also by mitochondrial oxidase (34). A possible participation of both NADPH and mitochondrial source seems to exist because the same inhibitory response was obtained in the presence of both apocynin, which blocks the association of p47phox cytosolic subunit with the NADPH oxidase membrane (35), and rotenone, which inhibits NADH dehydrogenase in mitochondrial complex I (36).

The time course of JAK2 activation by Ang II in the presence of high glucose appears to be a relatively late event when compared with previous studies performed in different cell types (14,37). However, in contrast with the quoted studies in which cells were preincubated in high glucose or normal glucose for long durations (up to 24 h) before the addition of Ang II, in our study the time course started after the addition of both high glucose and Ang II. Thus, it is conceivable that the late response of JAK2 phosphorylation may be due to the time required by glucose to increase ROS generation.

Therefore, these experiments, although they do not solve the question of whether in nonfailing human myocytes the capability of Ang II to induce ROS generation is limited or whether the ROS generated are promptly inactivated, clearly indicate that high glucose may allow Ang II to reach a threshold level of ROS necessary to activate JAK2.

The effects of Ang II in the presence of high glucose might also have clinical relevance owing to the early increase in Ang II cardiac generation in response to hemodynamic overload (38) or in asymptomatic patients in a very early phase of heart failure (20). The effects of a persistent increase of cardiac Ang II generation on growth factors production and collagen deposition (39) might thus be amplified in diabetic subjects. In particular, the close relationship between mechanical forces and cardiac Ang II generation stress the importance of reaching a low target of blood pressure in diabetic individuals.

Role of Ang II in glucose-induced JAK2 activation in failing myocytes. The present results show that failing myocytes, unlike those from nonfailing cells, respond to high glucose with JAK2 activation even in the absence of exogenous Ang II. The capability of AT1 antagonism to inhibit high glucose–induced JAK2 activation agrees with previous studies performed in rat cardiomyocytes that demonstrated the capability of glucose to induce Ang II formation (6). An important aspect revealed by the present study is that the same response is antagonized also by an ACE inhibitor. This result strongly suggests that Ang II–activating JAK2 is newly synthesized by myocytes and that ACE is a relevant source of Ang II formation in human failing myocytes. Other studies indicated that chymase rather than ACE is a major determinant for local Ang II generation in cardiac tissue (18). However, the expression of mRNA for ACE rather than chymase was reported to be increased in the myocardium of patients with heart failure (20). The limit of all these studies is mainly related to the absence of any information regarding isolated cardiomyocytes. The only study investigating this aspect was performed in animals (dogs) with experimental heart failure (pacing) and reported an increased expression of mRNAs for the RAS components regulating Ang II formation (angiotensinogen, renin, and ACE) in failing cardiomyoctes without any changes in chymase gene expression (40). The present study reveals for the first time that human ventricular cardiomyocytes express mRNAs for all the components of the RAS and that the progression to heart failure is characterized by marked overexpression of angiotensinogen and ACE with no changes in the expres-

TABLE 2
Characterization of Ang II receptors on isolated myocytes

	Nonfailing	Dilated cardiomyopathy
B_{max} Total (fmol/mg)	0.57 ± 0.21	0.60 ± 0.11
K_d (nmol/l)	0.19 ± 0.08	0.23 ± 0.10
AT1:AT2	79:21	73:27
B_{max} AT1 receptor (fmol/mg)	0.45 ± 0.17	0.44 ± 0.08
B_{max} AT2 receptor (fmol/mg)	0.12 ± 0.04	0.16 ± 0.05

Data are means ± SE.

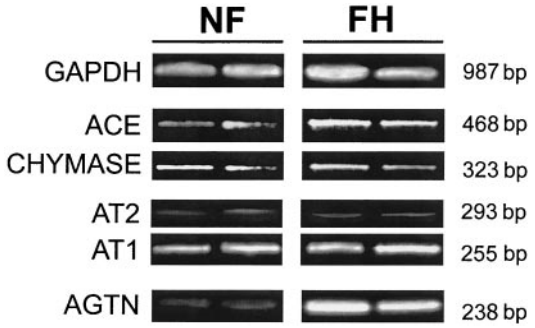

FIG. 8. Representative PCRs performed with total RNA isolated from myocytes obtained from two nonfailing hearts (NF) and two failing hearts (FH) using specific primers for GAPDH, ACE, chymase, AT1 and AT2 receptor subtypes, and AGTN.

sion of chymase. Although these results do not exclude that both enzymes may be involved in Ang II formation in failing myocytes, the comparable effect of AT1 antagonist and ACE inhibition on JAK2 phosphorylation suggests that in this condition ACE may represent the major pathway for myocyte Ang II formation.

Experiments performed with the different ROS inhibitors indicate that also in failing myocytes ROS generation mediates high glucose–induced JAK2 phosphorylation. Therefore, also in failing adult myocytes, ROS generation is a determinant for Ang II to induce JAK2 activation. The enhanced ROS generation might be particularly relevant in human failing hearts where NADPH oxidase, which is recognized as a major source of ROS, is known to be overactivated (41).

The second relevant difference observed in failing myocytes is that, at variance with nonfailing cells, Ang II induces JAK2 activation also in the presence of normal glucose concentration. The two Ang receptor subtypes mediate opposite intracellular effects in cardiomyocytes because AT1 was reported to activate NADPH oxidase (41–43), JAK2 (9,10,27), and MAPK phosphorylation (44), whereas AT2 was shown to antagonize AT1 effects by activating MAPK-1 phosphatase (29,30). The different pattern of response between failing and nonfailing cells appears undue to a different expression of Ang II receptors, because failing and nonfailing cells share the same absolute number and relative AT1–to–AT2 receptor ratio at both protein and mRNA levels. On the other hand, the different JAK2 response to Ang II stimulation is unlikely due to the participation of a different source of ROS. Antioxidant enzymes are decreased in the failing heart (33,41), so that it cannot be excluded that high glucose–induced superoxide generation may be increased in myocytes isolated from failing hearts.

In conclusion, although we did not investigate the activation of different STATs (signal transducer activators of transcription) following JAK2 phosphorylation in either nonfailing or failing hearts, our results may have relevant clinical implications. The capability of high glucose to allow Ang II to activate an important pathway for myocyte hypertrophy in nonfailing hearts may offer a reason for the importance of the strict blood pressure control in diabetic individuals. On the other hand, the capability of glucose to directly enhance Ang II release in failing hearts and to activate a pathway that is also related to intracellular signaling of inflammation indicates that high glucose can directly amplify diabetic cardiac complications.

REFERENCES

1. Galderisi M, Anderson KM, Wilson PW, Levy D: Echocardiographic evidence for the existence of a distinct diabetic cardiomyopathy (The Framingham Heart Study). *Am J Cardiol* 68:85–89, 1991
2. Devereux RB, Roman MJ, Paranicas M, O'Grady MJ, Lee ET, Welty TK, Fabsitz RR, Robbins D, Rhoades ER, Howard BV: Impact of diabetes on cardiac structure and function: the Strong Heart Study. *Circulation* 101:2271–2276, 2000
3. Poirier P, Garneau C, Marois L, Bogaty P, Dumesnil J-G: Diastolic dysfunction in normotensive men with well-controlled type 2 diabetes: importance of maneuvers in echocardiographic screening for preclinical diabetic cardiomyopathy. *Diabetes Care* 24:5–10, 2001
4. Frustaci A, Kajstura J, Chimenti C, Jakoniuk I, Leri A, Maseri A, Nadal-Ginard B, Anversa P: Myocardial cell death in human diabetes. *Circ Res* 87:1123–32, 2000
5. Kannel WB, Hjortland M, Castelli WP: Role of diabetes in congestive heart failure: the Framingham Study. *Am J Cardiol* 34:29–34, 1974
6. Fiordaliso F, Leri A, Cesselli D, Limana F, Safai B, Nadal-Ginard B, Anversa P, Kajstura J: Hyperglycemia activates p53 and p53-regulated genes leading to myocyte cell death. *Diabetes* 50:2363–2375, 2001
7. Ueyama T, Kawashima S, Sakoda T, Rikitake Y, Ishida T, Kawai M, Yamashita T, Ishido S, Hotta H, Yokoyama M: Requirement of activation of the extracellular signal-regulated kinase cascade in myocardial cell hypertrophy. *J Mol Cell Cardiol* 32:947–960, 2000
8. Yue TL, Gu JL, Wang C, Reith AD, Lee JC, Mirabile RC, Kreutz R, Wang Y, Maleeff B, Parsons AA, Ohlstein EH: Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. *J Biol Chem* 275:37895–37901, 2000
9. Marrero MB, Schieffer B, Li B, Sun J, Harp JB, Ling BN: Role of Janus kinase/signal transducer and activator of transcription and mitogen-activated protein kinase cascades in angiotensin II- and platelet-derived growth factor-induced vascular smooth muscle cell proliferation. *J Biol Chem* 272:24684–24690, 1997
10. Schieffer B, Luchtefeld M, Braun S, Hilfiker A, Hilfiker-Kleiner D, Drexler H: Role of NAD(P)H oxidase in angiotensin II-induced JAK/STAT signaling and cytokine induction. *Circ Res* 87:1195–1201, 2000
11. McWhinney CD, Hunt RA, Conrad KM, Dostal DE, Baker KM: The type I angiotensin II receptor couples to Stat1 and Stat3 activation through Jak2 kinase in neonatal rat cardiac myocytes. *J Mol Cell Cardiol* 29:2513–24, 1997
12. McWhinney CD, Dostal D, Baker K: Angiotensin II activates Stat5 through Jak2 kinase in cardiac myocytes. *J Mol Cell Cardiol* 30:751–761, 1998
13. Kodama H, Fukuda K, Pan J, Makino S, Sano M, Takahashi T, Hori S, Ogawa S: Biphasic activation of the JAK/STAT pathway by angiotensin II in rat cardiomyocytes. *Circ Res* 82:244–50, 1998
14. Amiri F, Shaw S, Wang X, Tang J, Waller JL, Eaton DC, Marrero MB: Angiotensin II activation of the JAK/STAT pathway in mesangial cells is altered by high glucose. *Kidney Int* 61:1605–1616, 2002
15. Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 48:1–9, 1999
16. Dell'Italia LJ, Meng QC, Balcells E, Straeter-Knowlen IM, Hankes GH, Dillon R, Cartee RE, Orr R, Bishop SP, Oparil S, Elton TS: Increased ACE and chymase-like activity in cardiac tissue of dogs with chronic mitral regurgitation. *Am J Physiol* 269:H2065–H2073, 1995
17. Hoit BD, Shao Y, Kinoshita A, Gabel M, Husain A, Walsh RA: Effects of angiotensin II generated by an angiotensin converting enzyme-independent pathway on left ventricular performance in the conscious baboon. *J Clin Invest* 95:1519–1527, 1995
18. Urata H, Boehm KD, Philip A, Kinoshita A, Gabrovsek J, Bumpus FM, Husain A: Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart. *J Clin Invest* 91:1269–1281, 1993
19. Zisman LS, Abraham WT, Meixell GE, Vamvakias BN, Quaife RA, Lowes BD, Roden RL, Peacock SJ, Groves BM, Raynolds MV: Angiotensin II formation in the intact human heart: predominance of the angiotensin-converting enzyme pathway. *J Clin Invest* 96:1490–1498, 1995
20. Neri Serneri GG, Boddi M, Cecioni I, Vanni S, Coppo M, Papa ML, Bandinelli B, Bertolozzi I, Polidori G, Toscano T, Maccherini M, Modesti PA: Cardiac angiotensin II formation in the clinical course of heart failure and its relationship with left ventricular function. *Circ Res* 88:961–968, 2001
21. Ohmichi N, Iwai N, Kinoshita M: Expression of angiotensin converting enzyme and chymase in human atria. *J Hypertens* 15:935–943, 1997
22. Asano K, Dutcher DL, Port JD, Minobe WA, Tremmel KD, Roden RL, Bohlmeyer TJ, Bush EW, Jenkin MJ, Abraham WT, Raynolds MV, Zisman LS, Perryman MB, Bristow MR: Selective downregulation of the angiotensin II AT1-receptor subtype in failing human ventricular myocardium. *Circulation* 95:1193–1200, 1997
23. Francis GS: ACE inhibition in cardiovascular disease. *N Engl J Med* 342:201–202, 2000
24. Sadoshima J, Montagne O, Wang Q, Yang G, Warden J, Liu J, Takagi G, Karoor V, Hong C, Johnson GL, Vatner DE, Vatner SF: The MEKK1-JNK pathway plays a protective role in pressure overload but does not mediate cardiac hypertrophy. *J Clin Invest* 110:271–279, 2002
25. Hirota H, Chen J, Betz UA, Rajewsky K, Gu Y, Ross J Jr, Muller W, Chien KR: Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. *Cell* 97:189–198, 1999
26. Fukuzawa J, Booz GW, Hunt RA, Shimizu N, Karoor V, Baker KM, Dostal DE: Cardiotrophin-1 increases angiotensinogen mRNA in rat cardiac

myocytes through STAT3: an autocrine loop for hypertrophy. *Hypertension* 35:1191–1196, 2000
27. Ali MS, Sayeski PP, Dirksen LB, Hayzer DJ, Marrero MB, Bernstein KE: Dependence on the motif YIPP for the physical association of Jak2 kinase with the intracellular carboxyl tail of the angiotensin II AT1 receptor. *J Biol Chem* 272:23382–23388, 1997
28. Hefti MA, Harder BA, Eppenberger HM, Schaub MC: Signaling pathways in cardiac myocyte hypertrophy. *J Mol Cell Cardiol* 29:2873–2892, 1997
29. Huang XC, Richards EM, Sumners C: Mitogen-activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors. *J Biol Chem* 271:15635–15641, 1996
30. Horiuchi M, Hayashida W, Kambe T, Yamada T, Dzau VJ: Angiotensin type 2 receptor dephosphorylates Bcl-2 by activating mitogen-activated protein kinase phosphatase-1 and induces apoptosis. *J Biol Chem* 272:19022–19026, 1997
31. Simon AR, Rai U, Fanburg BL, Cochran BH: Activation of the JAK-STAT pathway by reactive oxygen species. *Am J Physiol* 275:C1640–C1652, 1998
32. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW: Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 74:1141–1148, 1994
33. Takano H, Zou Y, Hasegawa H, Akazawa H, Nagai T, Komuro I: Oxidative stress-induced signal transduction pathways in cardiac myocytes: involvement of ROS in heart diseases. *Antioxid Redox Signal* 5:789–794, 2003
34. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787–790, 2000
35. Stolk J, Hiltermann TJ, Dijkman JH, Verhoeven AJ: Characteristics of the inhibition of NADPH oxidase a ctivation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol* 11:95–102, 1994
36. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ: Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem* 278:36027–36031, 2003
37. Shaw S, Wang X, Redd H, Alexander GD, Isales CM, Marrero MB: High glucose augments the angiotensin II-induced activation of JAK2 in vascular smooth muscle cells via the polyol pathway. *J Biol Chem* 278:30634–30641, 2003
38. Modesti PA, Vanni S, Bertolozzi I, Cecioni I, Polidori G, Paniccia R, Bandinelli B, Perna A, Liguori P, Boddi M, Galanti G, Neri Serneri GG: Early sequence of cardiac adaptations and growth factor formation in pressure- and volume-overload hypertrophy. *Am J Physiol Heart Circ Physiol* 279:H976–H985, 2000
39. Modesti PA, Vanni S, Bertolozzi I, Cecioni I, Lumachi C, Perna AM, Boddi M, Gensini GF: Different growth factor activation in the right and left ventricles in experimental volume overload. *Hypertension* 43:101–108, 2004
40. Barlucchi L, Leri A, Dostal DE, Fiordaliso F, Tada H, Hintze TH, Kajstura J, Nadal-Ginard B, Anversa P: Canine ventricular myocytes possess a renin-angiotensin system that is upregulated with heart failure. *Circ Res* 88:298–304, 2001
41. Heymes C, Bendall JK, Ratajczak P, Cave AC, Samuel JL, Hasenfuss G, Shah AM: Increased myocardial NADPH oxidase activity in human heart failure. *J Am Coll Cardiol* 41:2164–2171, 2003
42. Zafari AM, Ushio-Fukai M, Minieri CA, Akers M, Lassegue B, Griendling KK: Arachidonic acid metabolites mediate angiotensin II-induced NADH/NADPH oxidase activity and hypertrophy in vascular smooth muscle cells. *Antioxid Redox Signal* 1:167–179, 1999
43. Touyz RM, Chen X, Tabet F, Yao G, He G, Quinn MT, Pagano PJ, Schiffrin EL: Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries: regulation by angiotensin II. *Circ Res* 90:1205–1213, 2002
44. Touyz RM, Schiffrin EL: Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev* 52:639–672, 2000