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Gender-Related Differences in Basal and Hypoxia-Induced Activation of Signal Transduction Pathways Controlling Cell Cycle Progression and Apoptosis, in Cardiac Fibroblasts

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Previously we showed that cardiac fibroblasts are cellular targets of estrogen and that there are significant differences in proliferative response of male and female cardiac fibroblasts under hypoxia, a condition of myocardial ischemia. Here, we tested the hypothesis that signaling pathways that control cell cycle progression and apoptosis in cardiac fibroblasts may be activated in a gender-specific manner. Cardiac fibroblasts from adult, age-matched male and female rat heart were exposed to hypoxia (2% O_2) and normoxia. Western analysis of cell lysate was used to compare the level of basal and hypoxia-induced expression of signal transduction proteins, known to control cell cycle progression and cell death. Hypoxia led to significant activation of MAP (mitogen-activated protein) kinase and Jun kinase pathways, as shown by phosphorylated extracellular signal-regulated kinase (ERK1/2) and Jun kinase isotypes in male cells but this effect was modest in female cells. Male cells expressed higher levels of basal expression for transcription factors c-jun and NF-kB as well as the inhibitor of NF-kB (Ik-B). Although hypoxia did not induce changes in the level of c-Jun in either cell type, it moderately increased the level of NF-kB in male cells but led to its decrease in female cells. Basal and hypoxia-induced expression of cyclin D1, c-fos, and PCNA seemed to be comparable in both male and female cells. However, hypoxia-induced activation of cyclin B1, which occurred in both cells, was stronger in female cells. Basal expression of apoptosis-associated transcription factor, p53, was comparable in both cells. However, under hypoxia, there was an increase in the p53 level only in female cells. Although female cells showed higher basal expression for survival-associated protein, Bcl-2, the level of this protein remained unchanged under hypoxia in both cells. Together, these data demonstrate differences in basal and hypoxia-induced expression of proteins with an established role in cell cycle progression and apoptosis in male and female cardiac fibroblasts. These differences may further point to gender-related differences in signal transduction pathways that control the proliferative response of those cells under hypoxia.

Key Words: Hypoxia; ischemic heart disease; proliferation; estrogen; gender; fibroblasts.

Introduction

Although underlying mechanisms of gender-based predisposition to heart diseases are vastly unknown, gender-related differences in the response of cardiac cells to stress stimuli causing heart failure are established. Studies from our laboratory and by other investigators provide clear evidence that cardiac cells are targets of sex hormones, and their biological response under stressful stimuli can be regulated by their exposure to those hormones. Cardiac fibroblasts are the source of extracellular matrix, and their proliferation, which is observed in infarct areas of the myocardium, is an underlying mechanism of both physiological and pathological remodeling of the extracellular matrix in the heart. We and others have established that cardiac fibroblasts are cellular targets for estrogen *(1–4)*. We also showed that, in female cells, estrogen increases proliferative response via estrogen receptor- and mitogen-actived protein (MAP) kinase-dependent mechanisms *(1)*.

Proliferation of cardiac fibroblasts is regulated by neurohormonal and hemodynamic stimuli, including hypoxia, a feature of myocardial ischemia *(5–10)*. Hypoxia elicits autoprotective responses that can contribute to mechanisms leading to cell death/proliferation. Previously, we showed that hypoxia regulates both basal and growth-factor-induced DNA synthesis in human cardiac fibroblasts *(5)*. More recently, we demonstrated that although female cells are resistant to hypoxia-induced inhibition in DNA synthesis, male cells are susceptible *(6)*. In those studies, we also showed that intracellular pathways involving tyrosine phosphorylation are involved in the response of both cells and that estrogen,via estrogen receptor-dependent mechanisms, differentially alters the response of male and female cells to hypoxia *(6)*. Together, those findings form the basis for our hypothesis that differences may exist in the basal and

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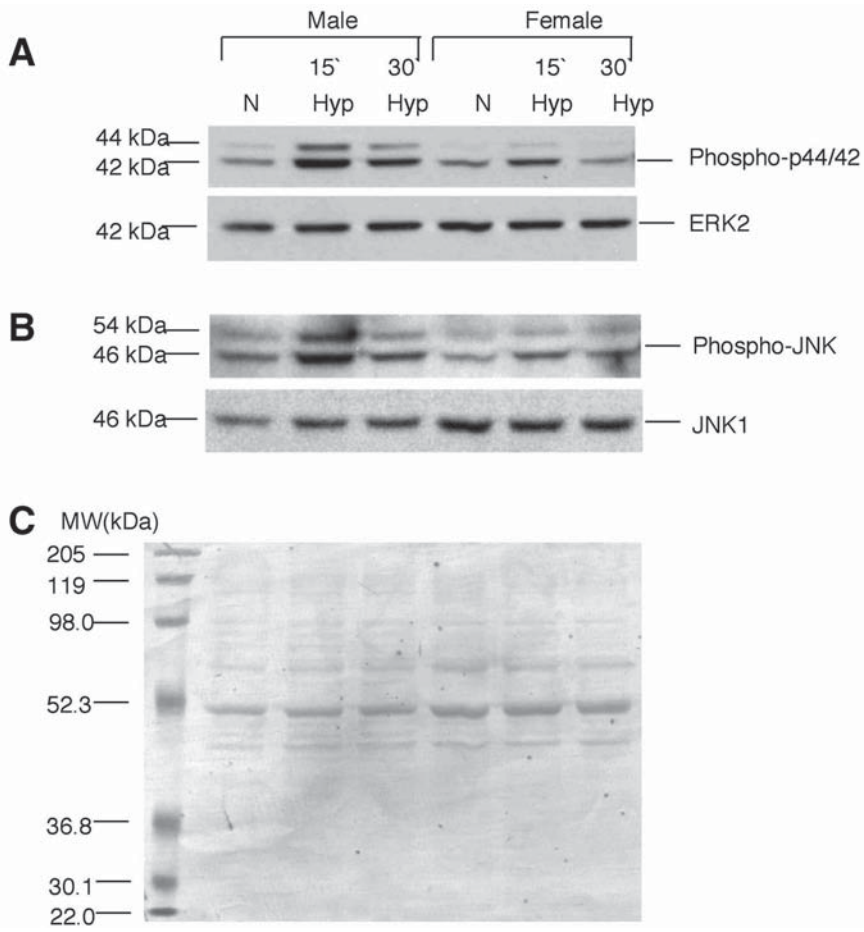

Fig. 1. Western analysis of cell lysate for phosphorylated MAP and Jun kinase proteins. Cell lysate was prepared from confluent quiescent cardiac fibroblasts in ambient conditions and under hypoxia (2% oxygen) as described in the Materials and Methods section. Aliquots of cell lysate (30 μg protein) were electrophoresed, electrophoretically transferred to a membrane, and then incubated with anti-phospho ERK1/2 antibody (**A**, upper row). The blots were then stripped and reincubated with anti-native ERK2 (**A**, lower row), phospho Jun kinase antibody (**B**, upper row), and native Jun kinase (**B**, lower row). Protein–antibody complexes were visualized by enhanced chemiluminescence as described in the Materials and Methods section. In (**C**), the photograph of global protein staining of the same blot (after removal of antibodies) with Coomassie blue is provided. These photographs are representatives of two independent experiments.

hypoxia-induced activation of signal transduction pathways controlling cell cycle progression and cell death in male and female cardiac fibroblasts. The goal of the present study was to test this hypothesis by comparing basal and hypoxia-induced expression of proteins with the established role in controlling cell cycle progression and cell death.

Results

Basal and Hypoxia-Induced Activity of MAP Kinase and Jun Kinase Pathways

The activation of MAP kinase and Jun kinase pathways in response to environmental stimuli is a central event leading to changes in the expression and activation of an array of transcription factors involved in the regulation of cell cycle progression and apoptosis. To determine differences in the activation of these pathways in male and female cells in response to hypoxia, we determined the abundance of phosphorylated ERK1/2 and phosphorylated Jun kinase in total cellular proteins obtained from male and female cardiac fibroblasts after exposure to hypoxia. The results pointed to differences between the responses of the two cells. In male cells, a 15-min exposure to hypoxia led to a sharp increase in the abundance of phosphorylated ERK1 and ERK2 compared with that in cells under normoxia. The level of expression for phosphorylated ERK1/2 declined toward normal levels but remained elevated during 30 min of hypoxia. In female cells on the other hand, at 15 min of hypoxia, only phosphorylated ERK2 was elevated, but returned to normal levels at 30 min of hypoxia (Fig. 1). During the same periods, the level of native ERK2 remained unchanged in both cell types (Fig. 1). Similarly, there were differences in hypoxia-induced activation of Jun kinase in male and female

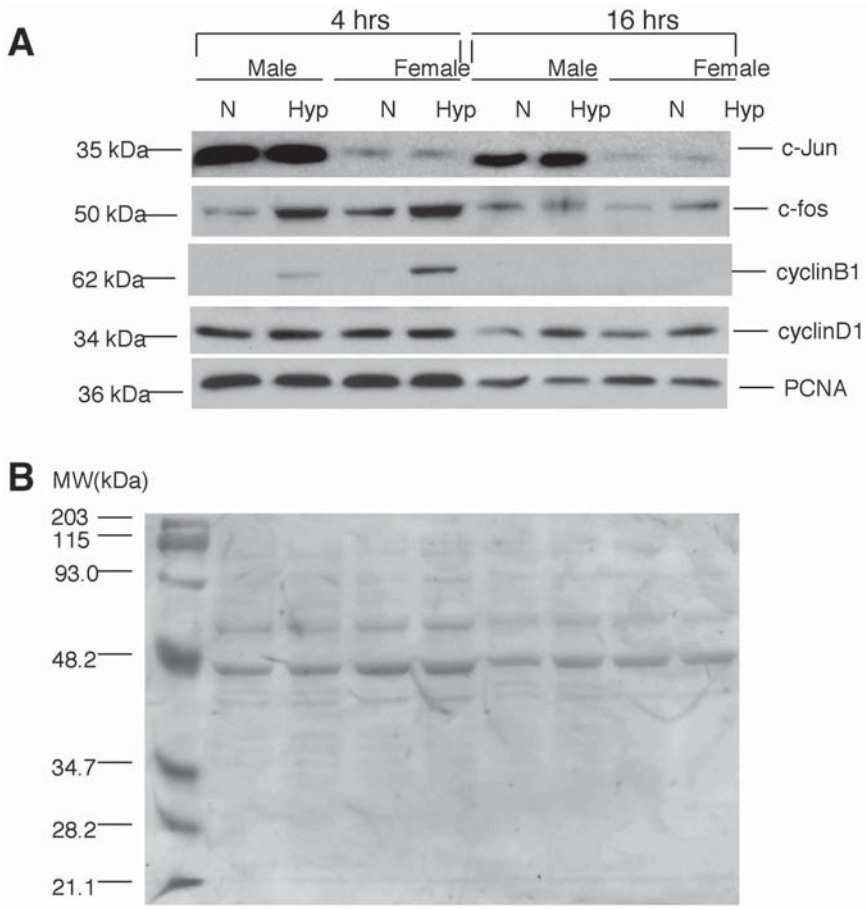

Fig. 2. Western analysis of cell lysate for transcription factors involved in cell cycle progression. Cell lysate was prepared from confluent quiescent cardiac fibroblasts in ambient conditions and under hypoxia (2% oxygen) as described in the Materials and Methods section. Aliquots of cell lysate (35 μg protein) were electrophoresed, electrophoretically transferred to a membrane, and then incubated serially with anti-c-Jun, c-fos, cyclin B1, cyclin D1, and PCNA antibodies (**A**). Protein–antibody complexes were visualized by enhanced chemiluminescence as described in the Materials and Methods section. In (**B**), the photograph of global protein staining of the same blot (after removal of antibodies) with Coomassie blue is provided. These photographs are representatives of two independent experiments.

cells. In male cells, at 15 min of hypoxia, the abundance of both 54- and 46-kDa phosphorylated proteins increased compared to that in cells under normoxia. The level of both declined with time toward the normal level (Fig. 1). In female cells, however, there was only a modest increase in the level of 46 kDa phosphorylated protein at 15 min, which returned to normal levels at 30 min. The level of native Jun kinase remained unchanged during the same periods in both cells (Fig. 1).

Basal and Hypoxia-Induced Expression of Transcription Factors Associated with Cell Cycle Progression

To determine differences in the hypoxia-induced activation of proteins downstream of MAP kinase and Jun kinase pathways, we determined the level of c-Jun, c-fos, cyclin B1, cyclin D1, and PCNA expression in male and female cardiac fibroblasts after exposure to hypoxia. The results showed that the basal level of c-Jun expression in male cells was significantly higher than that in female cells. Neither early (4 h) nor prolonged (16 h) exposure to hypoxia led to increased levels of c-Jun expression in either male or female cells (Fig. 2). In contrast, the basal level of c-fos expression was comparable in both male and female cells and hypoxia led to increased expression of c-fos in both cells (Fig. 2). The basal and hypoxia-induced levels of cyclin D1 expression were also comparable in both male and female cells, whereas the hypoxia-induced activation of cyclin B1, which occurred in both cells, was stronger in female cells (Fig. 2). The basal level of PCNA, another transcription factor with a key role in cell proliferation, was comparable in both male and female cells. However, in male cells, there was a modest hypoxia-induced decrease in the level of PCNA, whereas in female cells, the level of this protein remained unchanged (Fig. 2).

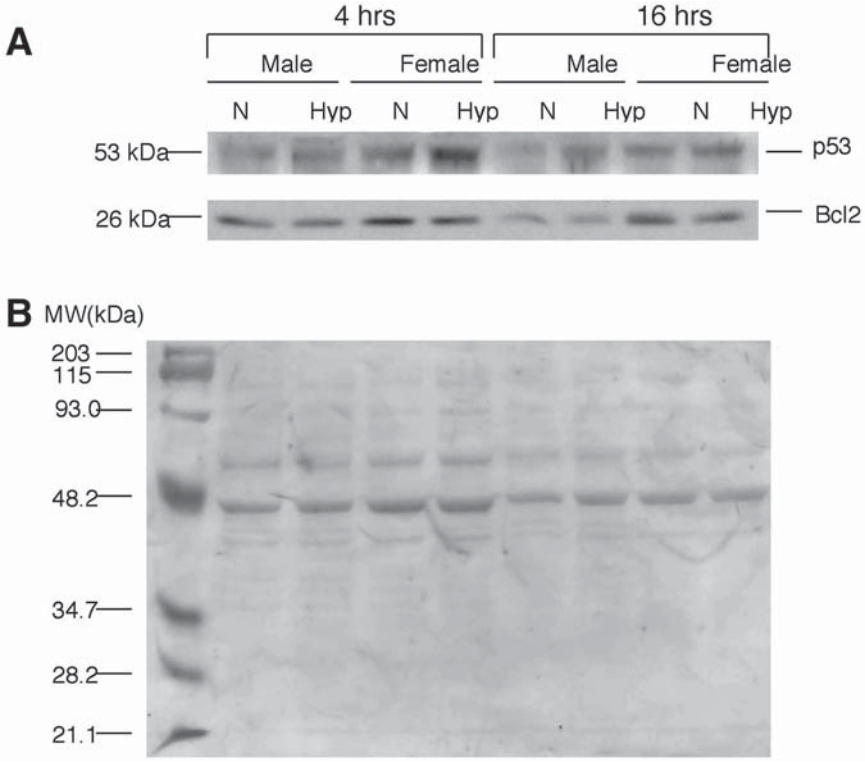

Fig. 3. Western analysis of cell lysate for apoptosis and survival-associated proteins. Cell lysate was prepared from confluent quiescent cardiac fibroblasts in ambient conditions and under hypoxia (2% oxygen) as described in the Materials and Methods section. Aliquots of cell lysate (35 µg protein) were electrophoresed, electrophoretically transferred to a membrane, and then serially incubated with anti-p53 and anti-Bcl-2 antibodies (**A**). Protein–antibody complexes were visualized by enhanced chemiluminescence as described in the Materials and Methods section. In (**B**), the photograph of global protein staining of the same blot (after removal of antibodies) with Coomassie blue is provided. These photographs are representatives of two independent experiments.

Basal and Hypoxia-Induced Expression of Transcription Factors Associated with Apoptosis and Cell Survival

To gain insight into gender-related differences in hypoxia-induced expression of transcription factors that control cell death and survival, we determined the level of p53 and Bcl-2 in cell lysates obtained from cells under hypoxia and those under normoxia, by Western analysis. The results showed an increase in the expression of p53 in female cells under hypoxia, whereas in male cells, the level of this transcription factor did not change (Fig. 3). The level of Bcl-2 under hypoxia did not change in male or female cells. However, the basal expression of Bcl2 was comparatively higher in female cells (Fig. 3).

Basal and Hypoxia-Induced Expression of Hif-1α and NF-kB

Hif-1α, whose expression is controlled by oxygen concentration, is the determinant factor in hypoxia-induced transcriptional activation of genes-encoding proteins that mediate adaptive responses to hypoxia. NF-kB is a protein with DNA-binding and transcription-activation capacities that ubiquitously regulates expression of various stress-related genes, including those activated by hypoxia. We determined the expression of both transcription factors in male and female cells under hypoxia. The results of hif-1α immunoprecipitation and Western analysis of immunocomplexes showed that hif-1α was induced in response to hypoxia in both male and female cells (Fig. 4). The results of Western analysis of cell lysate showed that although the level of NF-kB remains unchanged after short (4 h) exposure to hypoxia in both male and female cells, prolonged hypoxia (16 h) led to a decrease in NF-kB in female cells but not in male cells. Furthermore, the basal level of NF-kB expression was higher in male cells compared with that in female cells (Fig. 4). Similarly, the basal level of an inhibitor of NF-kB, Ik-B, was higher in male cells compared with female cells, but remained unchanged under hypoxia in both male and female cells (Fig. 4).

Discussion

The findings of this study demonstrate that hypoxia elicits different responses in cardiac fibroblasts obtained from the male and female hearts. These findings also suggest that the gender-related difference likely stems from apparent inherent differences in biological properties of male and female

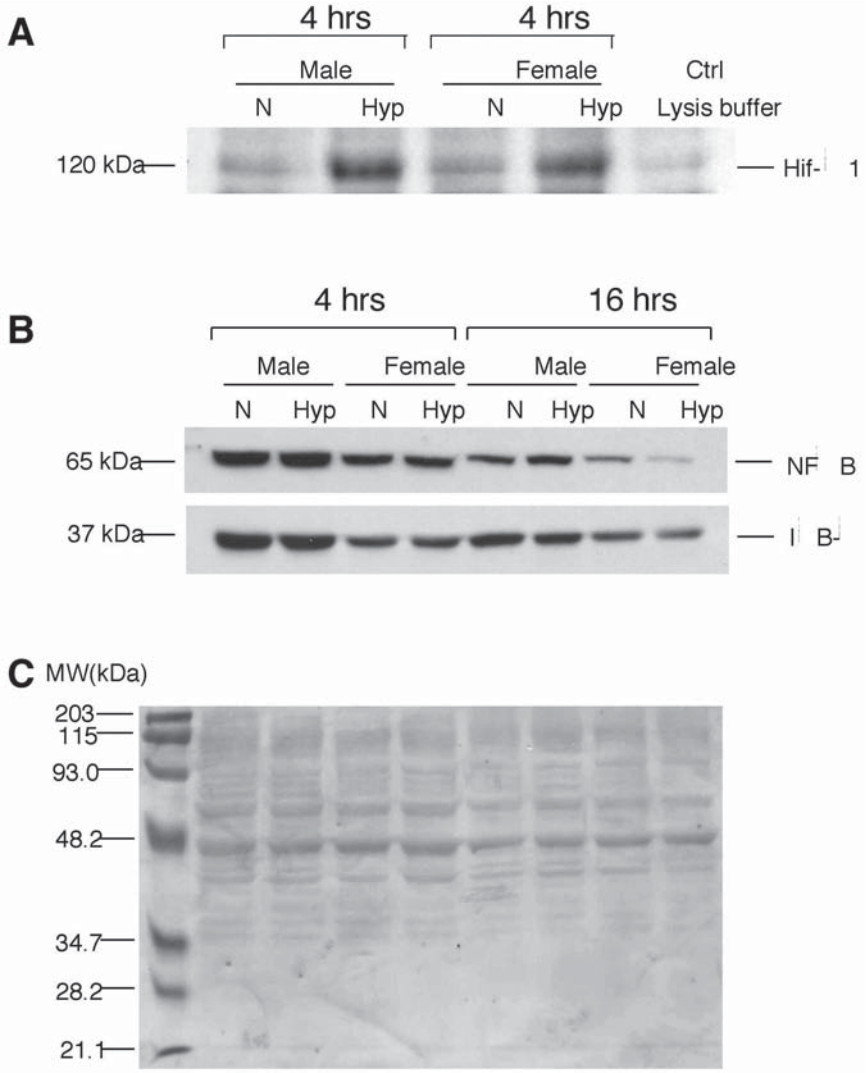

Fig. 4. Western analysis of Hif-1α immunocomplexes and cell lysate for transcription factors Hif-1α, NF-kB, and its inhibitor, Ik-B. Cell lysate was prepared from confluent quiescent cardiac fibroblasts in ambient conditions and under hypoxia (2% oxygen) as described in the Materials and Methods section. Aliquots of cell lysate (130 μg protein) were used for immunoprecipitation of hif-1α (**A**) and processed as described in the Materials and Methods section. In (**B**), aliquots of cell lysate (35 μg protein) were electrophoresed, electrophoretically transferred to a membrane, and then serially incubated with anti-NF-kB and Ik-B antibodies. Protein–antibody complexes were visualized by enhanced chemiluminescence as described in the Materials and Methods section. In (**C**), the photograph of global protein staining of the same blot (after removal of antibodies) with Coomassie blue is provided. These photographs are representatives of two independent experiments.

cells. The key items of evidence supporting this notion are as follows: First, the hypoxia-induced activation of signal transduction pathways controlling the cell cycle, including MAP kinase and Jun kinase pathways is more pronounced and persistent in male cells than in female cells; second, the basal expression of transcription factors that control the cell cycle, including c-Jun and NF-kB, is higher in male cells compared to that in female cells; third, the hypoxia-induced activation of cyclin B1 is more pronounced in female cells than in male cells; fourth, apoptosis-associated transcription factor, p53, is induced only in female cells and not in male cardiac fibroblasts; fifth, although hypoxia does not induce significant changes in the level of Bcl-2, the basal level of this survival-associated transcription factor is higher in female cells compared with that in male cells. Although the underlying mechanisms of these differences remain to be determined, they may, at least in part, reflect the variations that exist between male and female cardiac fibroblasts with respect to their receptors for estrogen and androgens. These receptors are DNA-binding proteins and act as transcription factors either as repressor or activators of transcription. It is now established that in addition to classical target tissues, estrogen and androgens act on a wide spectrum of tissues, including the cardiovascular system. As previously shown by others and us, cardiac fibroblasts contain functional estrogen receptors *(1–4)*. We have also shown

that estrogen regulates proliferative response in cardiac fibroblasts and that the effects of estrogen in male and female cells were mediated via estrogen receptors as they were reversed by the pure anti-estrogen, ICI 182,780 *(6)*. In those studies, although Western analysis showed that hypoxia increases the level of estrogen receptor β in both male and female cells, the results of gel shift analysis showed gender-related differences in the binding activity of estrogen receptor. In male cells, hypoxia induced an increase in cytoplasmic ERE (estrogen response element)-binding activity and a decrease in nuclear ERE-binding activity. In female cells, however, both cytoplasmic and nuclear ERE-binding activities remained unchanged under hypoxia. Those data suggest that transcriptional activity of estrogen receptors, hence transcription of target genes in male and female cells, may vary under hypoxia. Androgen receptors may also play a similar role. Androgens have been implicated in the regulation of cell proliferation. Studies by Libby have shown that testosteron inhibits proliferation of cardiac fibroblasts *(12)*. Studies by Huber et al. *(13)* showed differential effects of estradiol and testosterone on Fas-dependent apoptosis in CD4+ Th2 cells by altering Bcl-2 expression. To understand the contribution of androgen receptors to the observed gender-related expression of cell cycle proteins, future studies must determine the gender-related differences in the basal and hypoxia-induced activity of androgen receptors in cardiac fibroblasts.

Mitogen-activated protein kinases play a crucial role in signal transduction pathways regulating cell proliferation, differentiation, and even apoptosis, in response to a vast array of extracellular stimuli. The findings of this study demonstrating differences in the intensity and duration of hypoxia-induced activation of ERK and Jun kinase pathways in male and female cells point to differences that may exist in hypoxia sensing. In the present study, the observed prolonged activation of ERK2 in male cells compared with that in female cells may seem inconsistent with our previously reported inhibition of DNA synthesis observed only in male cells under hypoxia *(6)*. Although ERK signaling is a prototype mechanism regulating cell proliferation, it has become increasingly evident that the activation of ERKs and nonproliferative responses of the cell are not mutually exclusive. This notion may particularly draw support from evidence demonstrating the high abundance of ERK2 in mature, nondividing neurons of the central nervous system *(14)*. Studies by Lin et al. *(15)* showed that in primary human diploid fibroblasts, premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling *(15)*. Lim et al. *(16)* also showed accumulation of phosphorylated ERK2 in cytoplasm of senescent human fibroblasts *(16)*. These findings may lead to the prospect that in nonproliferating cells, ERK signaling may act as coordinator and signal integrator. The notion of a cross-talk between estrogen receptors and ERK signaling is now supported by evidence obtained in a variety of target tissues. Estrogen regulates growth-related signaling pathways, including MAP kinase activation. In cardiac fibroblasts, we showed that estrogen regulates proliferative response via estrogen receptor- and MAP kinase-dependent mechanisms *(1)*. In cardiac myocytes, estrogen activates ERK1/2, c-Jun kinase, and p38 in a distinctive pattern *(17)*. On the other hand, the activity of the estrogen receptor is regulated by MAP kinase cascade. Kato et al. *(18)* have shown that the activity of the amino-terminal AF-1 of the estrogen receptor is modulated by the phosphorylation of its Ser118 through the MAP kinase signaling. Thus, the observed differential hypoxia-induced activation of ERK signaling in male and female cardiac fibroblasts may, in turn, cause differential phosphorylation, hence transcriptional activity of estrogen receptors in those cells.

Hypoxia is a powerful regulator of transcriptional and post-transcriptional gene expression for a variety of regulatory proteins. Those proteins, including growth factors, enzymes, and stress proteins, in turn, regulate proliferation and differentiation of cardiac cells and also act in concert to compensate for the hypoxia-induced metabolic deficit. It is established that hypoxia induces cellular responses by enhancing the expression of a master regulator, the hypoxia-inducible factor-1 (hif-1). Hif-1 is a basic helix–loop–helix transcription factor, whose expression is controlled by oxygen concentration *(19)*. It is also known that of the hypoxia-regulated genes involved in controlling cell cycle, those encoding p53 and Bcl-2 are hif-1α dependent *(20)*. The results of our present study showed that although there are gender-based differences in basal and hypoxia-induced expression of p53 and Bcl-2, the level of hypoxia-induced hif-1α expression was comparable in both male and female cells. To understand the role of hif-1α in gender-based differences in the expression of target genes, future studies need to determine differences that may exist in hif-1α-binding activity in male and female cells under hypoxia.

Of multiple cyclins necessary for the passage of cells from control points at the cell cycle, cyclin D1 and B1 are essential for the crucial checkpoints of G1/S and G2/M transition, respectively. The results of this study showed a comparable pattern of expression for cyclin D1 in male and female cells. Hypoxia led to an increased expression of cyclin B1 in both cells, but this effect was more pronounced in female cells compared with that in male cells. The stronger hypoxia-induced cyclin B1 in female cells is consistent with our previously reported findings that proliferative response of female cells is preserved under hypoxia *(6)*. However, in order to understand if the observed changes in cyclin B1 lead to actual mitosis, all molecular components involved in the regulation of cell cycle, including cyclin-dependent kinases and their inhibitors, must be considered. Mitosis in mammalian cells is controlled by cyclin-depedent kinase Cdk1 *(21)*. The binding of cyclin B1 to Cdk1 is a requisite for the activation of Cdk1. The Cdk1–cyclin B1 complex is entirely in the cytoplasm during prophase. In late prophase,

most of the Cdk1–cyclin B1 complex is translocated into the nucleus, after which the nuclear envelope breaks down *(21)*. The control of cyclin B1 localization is the subject of intense investigation and it is now known that the steady state of this protein is dependent on the relative rate of nuclear import and export. The findings of the present study do not determine whether the apparent increased expression of cyclin B1 in male and female cells under hypoxia is the result of enhanced transcription or its translocation from nucleus to cytoplasm.

The p53 tumor suppressor limits cellular proliferation by inducing cell cycle arrest and apoptosis in response to a variety of genotoxic and nongenotoxic signals. Hypoxia, anoxia, and hypoxy-mimetic chemicals including cobalt have been shown to induce p53 accumulation. Our present studies showed increased hypoxia-induced accumulation of p53 only in female cells after 4 h of hypoxia. The time-course of this accumulation is consistent with previously reported time-course of hypoxia-induced accumulation of p53 (for review, see ref. *22*). Accumulation of p53 depends on the factors affecting its degradation. June kinase and Mdm2 are responsible for p53 degradation by the proteasome pathway. The inactive Jun kinase binds to p53 and targets it for degradation. The activated Jun kinase, on the other hand, causes an increase in the level of p53 and its activation. In the present study, the hypoxia-induced activation of Jun kinase in female cells was less pronounced than that in male cells. Yet, female cells demonstrate higher levels of p53 in response to hypoxia, suggesting that mechanisms other than activation of Jun kinase may be involved in p53 accumulation in female cells. The p53 protein is a transcription factor and its activation following its accumulation leads to transcriptional regulation of target genes. Three pathways have been proposed to account for hypoxia-induced activation of p53: (1) the hif-1-α signaling pathway; (2) changes in mitochondrial permeability leading to the release of free radicals and oxidative damage to DNA; (3) direct sensing of hypoxia by p53 in a manner similar to hif-1α *(22)*. Understanding the mechanisms by which p53 is induced in female cells in response to hypoxia and the reasons why male cells are resistant to this effect is of fundamental importance to the elucidation of gender-based differences in the survival of cardiac fibroblasts under hypoxia. Studies by Toda et al. *(23)* have shown that significant gender-related differences exist in the expression of genes associated with programmed cell death in lacrimal glands of autoimmune and normal mice and that some of these differences may be the result, at least in part, to the effect of androgens.

Bcl-2 is a dominant inhibitor of apoptotic cell death and its observed higher basal expression in female cells in this study is indeed compatible with our previously reported resistance of female cells under hypoxia *(6)*. The underlying mechanisms leading to the observed higher basal expression of Bcl-2 in female cells in this study were not determined. Findings of other investigators in Th2 cells that showed an increased expression of Bcl-2 with estradiol and its decreased expression with testosterone exposure *(13)* may, however, lend support to the notion that the response of cardiac fibroblasts to male and female hormones may play a role in regulating their Bcl-2 expression. Further studies to test this notion must determine the effect of estrogen and androgens on the expression of Bcl2 in those cells.

The activation of NF-kB is an early inducible event that participates in signal transduction pathways that mediate both cell cycle progression and cellular commitment to apoptosis. Inactive NF-kB in the cytosol is bound to the inhibitory protein, Ik-B. Phosphorylation of Ik-B by different regulatory kinases dissociates it from the NF-kB complex and causes NF-kB translocation to the nucleus, where it binds to DNA and activates the transcription of target genes. The results of this study showed a higher level of expression for both NF-kB and Ik-B in female cardiac fibroblasts compared with male cells. The functional interaction between estrogen receptor and NF-kB has been demonstrated both in vitro and in vivo *(24)* and it has been suggested that this interaction may contribute significantly to the cardioprotective effects of estrogen. Studies by Pelzer et al. *(25)* have shown that, in cardiac myocytes, estrogenic compounds inhibit NF-kB activity but not its expression. Although our results demonstrate higher basal expression of NF-kB in female cells, in order to understand the role of this transcription factor in gender-based differences in the response of cardiac fibroblasts to hypoxia, future studies must also determine the NF-kB-binding activities in male and female cells.

In summary, the results of this study that show differences in basal and hypoxia-induced gene expression in male and female cardiac fibroblasts may be used for further analyses aimed at elucidating some of the complex underlying mechanisms of gender-based differences in predisposition to and survival from ischemic heart disease. An understanding of the specific role of male and female hormones and the activity of their respective receptors along with differences that may exist in the binding activity of hif-1α and NF-kB in male and female cells as well as information on the cyclin-dependent kinases and their inhibitors is essential for elucidating mechanisms of gender-related differences in the response of cardiac fibroblasts to hypoxia.

Materials and Methods

Preparation of Cardiac Fibroblasts

Cultured cardiac fibroblasts were prepared as previously described *(6)*. Adult (8 wk) male and female Fischer 344 rats were anesthetized; hearts were excised, minced, and washed in phosphate-buffered saline (PBS). The tissue was then subjected to digestion at 35ºC by a mixture of 0.1% trypsin and 100 U/mL collagenase (Sigma) for 10 min by constant shaking. Isolated cells were pelleted at the end of each 10-min digestion period. Cells from the third to ninth digestion were plated on 100-mm culture dishes in Dulbecco's

modified Eagle medium (DMEM) and 10% fetal bovine serum (FBS) and incubated for 2 h at 37ºC in a humidified incubator with 10% CO_2. At the end of this period, the unattached cells were discarded, and attached cells were grown in DMEM + 10% FBS. The nature of cells was determined by immunofluorescence staining with anti-human factor VIII, anti-desmin, and anti-vimentin for identification of endothelial cells, muscle cells, and fibroblasts, respectively, as previously described *(11)*. In early passages *(1–2)*, only 1–2% of cells stained positively with anti-desmin and anti-factor VIII. All cells were stained positively with anti-vimentin antibody. For the experiments in this study, cells from passages 4–7 were used. At this stage, all cells stain negative for desmin or factor VIII and remain vimentin-positive.

Induction of Hypoxia

Hypoxia was induced as previously described *(5)* by reducing the concentration of oxygen in the incubator chamber (Forma Scientific) to 2% by infusion of nitrogen gas. The O_2 concentration was monitored using a Healthdyne Oxygen Concentration Monitor. An identical incubator chamber was infused with ambient air (21% oxygen, as determined by a Healthdyne Oxygen Concentration Monitor) and used for experiments under normoxic conditions. Both incubator chambers received an infusion of carbon dioxide to maintain a constant concentration of 10% for all experimental conditions.

Treatment of Cells

Cardiac fibroblasts were grown until 90–95% confluent; they were then serum-deprived for 18–24 h and exposed to hypoxia for 15 min, 30 min, 4 h, and 16 h. Matching control dishes were incubated under normoxia.

Western Analysis

The culture medium was aspirated and cells were rinsed three times with ice-cold PBS and lysed (scraped and passed three times through a 26-gage needle) in 0.5 mL of ice-cold lysis buffer (10 m*M* Tris-HCl, pH 7.4, 5 m*M* EDTA, 50 m*M* NaCl, 30 m*M* sodium pyrophosphate, 50 m*M* NaF, 0.1 m*M* sodium orthovanadate, 1 m*M* phenylmethylsulfonly fluoride, 2 m*M* benzamide, and 1% Triton X-100). Cell debris was removed by centrifugation (30 min at 16,000*g*). The supernatant was transferred to a new tube. The concentration of protein in cell lysate was measured using a BCA™ protein assay kit (Pierce, Rockford, IL) according to the manufacturer's guideline. Aliquots (30–35 µg protein) of cell lysate were mixed with one-half sample volume of 2X Laemmli buffer (62.5 m*M* Tris-HCl. pH 6.5, 2% sodium dodecyl sulfate [SDS], 20% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol), boiled for 5 min, and then electrophoresed in a 10% SDS–polyacrylamide gel. Proteins were electrophoretically transferred to an Immobilon™-P (Millipore Corp., MA) membrane, in accordance with the manufacturer's guidelines. The membrane was incubated overnight at 4ºC or for 1 h at room temperature with TBS (Tris-buffered saline) containing 3% dry milk. TBS contained (per liter) 8 g NaCl, 0.2 g KCl, and 3 g Tris base and was adjusted to pH 7.4 with HCl. The membrane was rinsed with TTBS (TBS containing 0.05% [v/v] Tween-20) three times (10 min each) and then incubated for 1 h in 10 mL of TTBS containing 0.2% nonfat dry milk and 0.5 µg/mL of rabbit polyclonal anti-c-jun and rabbit immunoaffinity purified anti-fos IgG (Upstate Biotechnology, Lake Placid, NY), polyclonal rabbit anti-cyclin B1, anti-Ik-B and anti-jun kinase antibodies, mouse monoclonal anti-cyclin D1, anti-PCNA, and anti-ERK2 antibodies (Santa Cruz Biotech, CA), mouse monoclonal anti-Bcl2 antibody (Neomarkers, Inc, Fermont, CA), rabbit polyclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr 204) and phospho-SAPK/JNK (Thr 183/Tyr 185) antibodies (Cell Signaling Technology, Inc. Beverly, MA), rabbit polyclonal anti-NF-kB antibody (Stress Gene Biotechnologies Corp. Victoria, BC, Canada) and a rabbit polyclonal anti-P53 antibody (Vector Laboratories, Inc., CA). The membrane was then rinsed with TTBS (15 min with three changes at 5-min intervals) and incubated for 1 h with TTBS containing 0.2% nonfat dry milk and 1:20,000 dilution of a horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Santa Cruz, CA). After rinsing with TTBS (15 min with three changes at 5-min intervals), bands corresponding to each protein–antibody complex were visualized by enhanced chemiluminescence using the ECL™ (Amersham Life Science, UK) kit according to the manufacturer's guidelines. Experiments were performed in duplicates.

Immunoprecipitation of Hif-1α Followed by Western Analysis

Aliquots of cell lysate (130 µg protein) were incubated with 1 µg of monoclonal mouse anti-hif-1α antibody (Novus Biologicals, Littleton, CO) and 25 µL protein A/G plus agarose (Oncogene Research Products, Cambridge, MA) with lysis buffer added to bring the total volume to 1 mL. Samples were immunoprecipitated overnight at 4ºC. After immunoprecipitation, samples were centrifuged in a microfuge for 5 min at 4ºC, the supernatant was aspirated, and the pellet was washed three times with lysis buffer and centrifuged each time for 5 min at 4ºC. The pellet was then resuspended in 35 µL of 1X SDS Laemmli buffer and boiled for 5 min. Samples were then electrophoresed and electrophoretically transferred to an Immobilon-P membrane (Millipore Corp., MA) and Western analysis proceeded as described earlier.

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