Regulation of *fos* and *jun* Immediate-Early Genes by Redox or Metabolic Stress in Cardiac Myocytes

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**Abstract** We have previously demonstrated coordinate inductions of *c-fos*, *c-jun*, *jun* B, and *jun* D in cardiac myocytes exposed to hypoxia for 2 to 4 hours. Induction of these transcripts occurred before any significant loss of intracellular ATP. In the present study, the origin of the signal(s) that regulates immediate-early gene induction was investigated by comparing the effects of hypoxia with those of the metabolic inhibitors cyanide, deoxyglucose and cyanide combined, and iodoacetic acid. Cyanide, an inhibitor of oxidative metabolism, closely mimicked the metabolic effects of hypoxia, with elimination of oxygen consumption, increased lactate production, and minimal decline in ATP levels under both conditions. Compared with hypoxia, cyanide mediated small transient inductions of *fos* and *jun* transcripts that followed a different time course. The combination of cyanide and deoxyglucose resulted in induction of lactate production as well as respiration, and ATP dropped rapidly to 20% of control levels. The loss of intracellular ATP was followed by fourfold inductions of *c-fos* and *c-jun* with minor changes in *jun* B and *jun* D transcript levels. Similarly, iodoacetic acid caused a major (90%) loss of ATP and irreversible cell damage as measured by leakage of creatine phosphokinase enzyme and loss of membrane arachidonic acid; ATP loss was followed by fivefold to sevenfold inductions of *c-fos*, *c-jun*, and *jun* B transcripts. We conclude that the hypoxic stress response in neonatal myocytes, which occurs before ATP depletion, cannot be fully accounted for by inhibition of oxidative metabolism or by factors related to metabolic switching. In contrast, the stress response associated with blockage of both aerobic and anaerobic energy metabolism coincides with and may be related to the loss of cellular ATP and structural cell damage. (*Circ Res.* 1994;74:679-686.)

**Key Words** • hypoxia • ischemia • proto-oncogene

Members of the *fos* and *jun* multigene families are induced rapidly and usually transiently in response to a range of diverse stimuli (reviewed in References 1 through 3). The Fos and Jun proteins constitute the transcription factor API.4-6 Induction of *fos* and *jun* as well as of other immediate-early-acting genes have been described in response to ischemia and reperfusion in the brain,7-10 kidney,11-13 liver,14,15 and heart.16 Neither the functional roles for these transient inductions nor the immediate initiating stimuli have been established. We have recently demonstrated that *c-fos*, *c-jun*, *jun* B, and *jun* D are all induced in cardiac myocytes exposed in vitro to severe hypoxia.17 This suggests that the hypoxic component of an ischemia/reperfusion stress can directly mediate the signal for induction of immediate-early genes, as opposed to secondary downstream factors that were previously suggested.7,11-15,18

A feature common to both hypoxia and ischemia in eukaryotic cells and tissues is the partial or complete switch from oxidative (respiratory) to anaerobic (glycolytic) energy metabolism.19-21 The anaerobic block of electron transport results in a global increase in the reduction of mitochondrial respiratory chain components and cofactors of oxidative phosphorylation, an effect that has been linked with the ensuing oxidative stress that can occur during ischemia/reperfusion.22 The switch also causes changes in the intracellular pools of other metabolic substrates, including glycogen, glucose, intermediate metabolites, fatty acids,23 and presumably glutathione.24 A number of these may be involved in second-messenger pathways.25-29 Metabolic switching in ischemic tissue also causes localized acidosis.19,20 Therefore, the regulation by oxygen availability of some cellular functions, including gene expression, could be a consequence of the biochemical changes that accompany metabolic switching. Alternatively (or additionally), the induction of immediate-early genes may be more directly coupled to other oxygen-sensitive redox states, independent of metabolic switching. Redox regulation of Jun activity and expression has been described previously.30-33 The possibility of molecular oxygen sensors in the myocardium as well as in other cells and tissues has also been suggested previously.34-40

If the signal(s) for immediate-early gene regulation by hypoxia is associated with metabolic switching and accompanying factors, then it should be possible to reproduce the effects by appropriate metabolic inhibition. To address this question, we measured the changes in expression of members of the *fos* and *jun* gene families in cardiac myocytes subjected to metabolic inhibitors and compared the changes with those observed during hypoxia. Although it was possible to closely reproduce the effects of hypoxia on metabolic switching as well as on intracellular ATP levels, metabolic inhibition did not reproduce the effects of hypoxia on *fos* and *jun* gene expression.

Received July 26, 1993; accepted December 17, 1993.

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Materials and Methods

Cell Culture

The isolation of cardiac myocytes from neonatal rat hearts has been described in detail elsewhere. Briefly, hearts from 30 to 40 pups were minced and subjected to serial trypsin digestion to release single cells. After the final digestion, the cells were washed and preplated for 0.5 hour in MEM with 5% fetal calf serum. Nonattached cells were replated in 60-mm Falcon dishes at \(4 \times 10^5\) cells per plate in the same medium with 0.1 mmol/L bromodeoxyuridine added for the first 4 days to reduce the background of nonmyocardial cells. Cultures were used for experiments after a further 2 or 3 days. These procedures reproducibly generate confluent cultures that contract synchronously at \(>300\) beats per minute, consistent with the normal beating rate of the intact newborn rat heart.

Hypoxia

Our methods for exposing cells to hypoxia have been described previously. Cultures were incubated in an environmental chamber (Anaerobic Systems, San Jose, Calif) at 37°C in a humidified atmosphere with 5% CO\(_2\), <0.2% oxygen, and the balance nitrogen. Oxygen tension was monitored with an oxygen electrode (Controls Katharobic, Philadelphia, Pa) inside the chamber.

Metabolic Inhibitors

To determine an appropriate concentration of KCN, increments from a 1 mol/L stock in MEM were added to suspensions of cardiac myocytes in sealed tubes while continuously monitoring oxygen consumption with the oxygen electrode. A final concentration of 1 mmol/L was determined to be the minimal concentration that completely eliminated oxygen uptake by these cells (data not shown). This concentration of KCN also mimicked the effects of hypoxia on lactic acid production. KCN and iodoacetic acid (IAA) were added directly to culture dishes as indicated from 1000x and 2000x stock solutions, respectively, dissolved in MEM, pH 7.2. Deoxyglucose was added from a 100x aqueous stock to 20 mmol/L. Cells were observed microscopically and recorded by using a Zeiss inverted microscope and COHU solid-state video camera linked to a Cell-Trak computer system as described previously.

RNA Analysis

RNA transcript levels were measured by Northern blots as described previously. Total RNA was isolated by solubilizing cells on the plate in 4 mol/L guanidinium thiocyanate (0.25 mL/10\(^6\) cells) and pelleting through cesium chloride. Agarose gels, blotting, and hybridizations were all as described previously. Complementary DNA probes including c-fos and c-jun (gifts of Tom Curran, Roche Institute of Molecular Biology, Nutley, NJ), jun B and jun D (purchased from the American Type Culture Collection), and B-actin were labeled by random priming (Prime-It II kit, Stratagene, Calif) to \(10^8\) cpm/\(\mu\)g DNA. RNA bands were analyzed by using a Lynx 4000 molecular biology workstation with application software for computerized Northern blot analysis (Applied Imaging Corp, Santa Clara, Calif). RNA loading on gels was monitored by ethidium bromide staining and by probing with a B-actin cDNA as described previously. Blots were only included if the ethidium stain demonstrated uniform ribosomal bands. Blots were probed with B-actin as the last probe of the series. All RNA quantification experiments were repeated at least five times, quantified signals were normalized with respect to a zero control time point, and the composite data were expressed in histogram form as the mean±SEM induction.

Lactate and Creatine Phosphokinase

Lactate and creatine phosphokinase (CPK) in the culture media were measured optically, as described previously, with kits from Sigma Chemical Co, St Louis, Mo. Briefly, 100-\(\mu\)L samples of medium were removed from each plate. For lactate determination, a portion of the sample was deproteinized with cold trichloroacetic acid (TCA), and lactate content of the supernatant was measured spectrophotometrically as described by the manufacturer. CPK was also determined spectrophotometrically by an enzyme-linked assay as described by the manufacturer.

Arachidonic Acid and ATP

The release of \(^{[3H]}\)arachidonic acid from myocyte membranes was measured as described previously. Briefly, cells were incubated for 20 hours in media containing 0.1 mcCi/mL \(^{[3H]}\)arachidonic acid (Amersham), after which time the radioactive medium was removed, rinsed with fresh media, and either exposed to metabolic inhibitors or to hypoxia. At appropriate time points, samples of media were removed, and the distribution of \(^{[3H]}\)arachidonic acid was determined by scintillation counting. At the end of the incubations, cells were harvested, and residual \(^{[3H]}\)arachidonic acid was determined. Arachidonic acid released was expressed as a percentage of total arachidonic acid incorporated.

ATP levels were measured using the luciferase-luciferin reaction (Sigma) after extraction of cells with 5% TCA exactly as described previously.

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

**Figure 1.** Graphs showing changes of lactate and ATP in response to KCN and hypoxia. Cells were incubated with 1 mmol/L KCN (left) or under hypoxia (right) as described in “Materials and Methods.” At the indicated times, plates were harvested with 5% trichloroacetic acid for ATP determinations, or samples of the extracellular medium were taken for lactate determination. ATP levels are expressed as the fraction of controls at time 0. Broken lines show aerobic untreated control cells. All results (mean±SEM) are from at least three separate experiments.
Results

Incubation of cells with 1 mmol/L KCN completely eliminated respiration as measured by oxygen uptake (not shown). Therefore, treatment of cells with KCN was at least as effective as hypoxia in inhibiting oxidative metabolism. Since under both conditions myocytes become dependent on glycolysis for ATP generation, extracellular lactate accumulation was measured as an index of the metabolic switch. Fig 1 shows that lactate production under hypoxia and after treatment with KCN was equivalent. Similarly, neither condition had a major effect on intracellular ATP, indicating that at least for the duration of these experiments, glycolysis was able to compensate for aerobic ATP production. Because KCN appeared to closely reproduce the effects of hypoxia on metabolic switching, we compared the effects of KCN on fos and jun immediate-early gene transcripts with those of hypoxia.

Fig 2 shows representative Northern blots (top panels) and composite profiles (bottom panel) of the transcript accumulations of c-fos, c-jun, jun B, and jun D from five experiments in which cardiac myocytes were exposed to 1 mmol/L KCN for 3 hours. Fig 2, top right and bottom, also shows the corresponding 2-hour hypoxia time points, included as a positive control. Whereas hypoxia-induced fos and jun transcripts peaked between 2 and 5 hours at between 5- and 10-fold (see also Reference 17), KCN treatment was associated with a very early transcript peak at ~15 minutes that involved ~3-fold induction and that was essentially reversed after 2 to 3 hours. Therefore, in contrast to their effects on the metabolic parameters described above, hypoxia and KCN had dissimilar effects on both the magnitude...
Treatment of cultures with deoxyglucose alone also caused transient twofold to threefold inductions of c-fos, c-jun, and jun D (n=5, data not shown). As the least perturbing intervention, the response to deoxyglucose is perhaps an indication of the high sensitivity of these genes to changes in metabolic flux.

IAA is a highly potent metabolic inhibitor that acts upstream from and inhibits both oxidative and glycolytic metabolic pathways. We used IAA treatment to examine extreme cellular stress that included the transition to lethal damage. Fig 5 shows the effects of IAA treatment on lactate accumulation, ATP, and cellular integrity as determined by arachidonic acid and CPK release. Measures of cell integrity are compared with those in the presence of the other metabolic inhibitors. As predicted, there was no induction of lactate production in the presence of IAA, and intracellular ATP levels fell to <10% of the control level within the first hour of exposure. In contrast to the other agents, IAA caused substantial loss of membrane arachidonic acid and intracellular CPK within the first 2 hours of exposure, indicating severe structural damage to the cells.

Transcript data from cultures treated with IAA are shown in Fig 6. After 1 and 2 hours of exposure to IAA, c-fos, c-jun, and jun B were all strongly induced. Interestingly, jun D was not affected. Transcript levels had declined at 4 hours, and further time points were not determined because of the severe structural damage to the cultures that could be observed microscopically (not shown; see References 38 and 45) and that was apparent from the biochemical assays (Fig 5, middle and bottom). Inhibition of aerobic and anaerobic energy pathways by IAA or KCN/deoxyglucose also caused a decline in β-actin transcript levels, particularly apparent at the later time points. This effect is presumably due to energy depletion. Fig 7 shows a typical set of ethidium bromide-stained gels demonstrating equivalent RNA loading in each case.

**Fig 3.** Graph showing changes of lactate and ATP in response to combined KCN and deoxyglucose. Conditions were as for Fig 1 except that 20 mmol/L deoxyglucose was included in the incubations.

**Fig 4.** Northern analysis of fos and jun expression in cells treated with potassium cyanide (CN) and deoxyglucose (DOG). The left panel is a representative blot, and the right panel is a bar graph showing transcript levels. Conditions were as for Fig 2 except that 20 mmol/L DOG was included in the incubations. Mean±SEM values on the right are the results of five separate experiments.
Discussion

In the present study, we have examined the possible contributions of metabolic stress, including ATP depletion to the hypoxia-mediated pathway of immediate-early gene induction in neonatal cardiac myocytes. We have demonstrated that the treatment of cells with the mitochondrial respiratory chain inhibitor KCN mediated the aerobic to anaerobic switching, which was quantitatively indistinguishable from subjecting the cultures to severe hypoxia in terms of lactate production and ATP generation. KCN (1 mmol/L) completely blocked oxygen uptake and was therefore at least as effective as hypoxia in inhibiting respiration. Lactate was generated under hypoxia at an average rate of 4.9±0.4 pmol/10^6 cells per hour and, after treatment with cyanide, 4.2±0.5 pmol/10^6 cells per hour. Neither treatment with KCN nor exposure to hypoxia caused significant change in intracellular ATP within the first 2 to 4 hours of treatment. This close correspondence of the effects of hypoxia and KCN on energy metabolism...
was not reflected in their effects on immediate-early gene expression. Hypoxia caused peak inductions of c-fos and c-jun of >8- and >4-fold, respectively, after 2 to 4 hours of exposure (Fig 2, top right; see Reference 17). KCN, on the other hand, mediated corresponding peak inductions of 3.1- and 2.8-fold, respectively, at 15 minutes, which declined slowly thereafter. Induction of these gene transcripts by KCN differed in both magnitude and time course from the corresponding effects of hypoxia. These results suggest that factors in addition to metabolic switching are responsible for the major immediate-early gene inductions caused by hypoxia.

Two major signal pathways have previously been shown to regulate fos/jun expression, including activation of protein kinases47-49 and changes of the redox state of Jun protein.30-33 In the latter pathway, the ability of Jun to bind DNA and thus transactivate transcription is determined by the redox state of a conserved cysteine residue located in the DNA binding domain of Fos and Jun.30 Only the reduced form of the Jun protein can bind DNA; thus, a more strongly reducing cellular environment would be predicted to favor transcriptional activation by Fos/Jun. Probably both kinase and redox factors contribute to the overall regulation of AP1-mediated transcription.

Whereas we have previously demonstrated a possible role for protein kinase C in immediate-early gene activation by hypoxia,17 it is clear that redox mechanisms may also be important. Both hypoxia exposure and KCN treatment cause increased reduction of mitochondrial electron transport chain components, cofactors, and coenzymes, including NADH and NADPH. Providing a pool of electron donors contributes to a more reduced state in the cell. In a converse but perhaps analogous situation, mitochondrial components have been shown to mediate free radical production and to contribute to oxidative damage under conditions of ischemia/reperfusion22 and hyperoxia.50 Possibly the induction of fos and jun by cyanide is associated with the reduced state of mitochondrial components, whereas hypoxia mediates additional reductive or phosphorylative changes unrelated to mitochondria. In pulmonary arteries, for example, hypoxia has been shown to modulate prostacyclin synthesis by directly regulating the activity of cyclooxygenase, an enzyme that catalyzes the oxidation of arachidonate to prostaglandin G2 (Reference 51 and references therein).

The effects of combined aerobic and anaerobic metabolic inhibition mediated immediate-early gene inductions that were more similar to hypoxia both in the magnitude and the time course of the response. Predictably, and in contrast with hypoxia, these conditions were accompanied by an immediate and major loss of intracellular ATP (Figs 3 and 5) and were also associated

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**Fig 6.** Northern analysis of fos and jun expression in cells treated with iodoacetic acid (IAA). The left panel is a representative blot, and the right panel is a bar graph showing transcript levels. Conditions were as for Fig 2 except that cells were treated with 50 mmol/L IAA for the indicated times. Mean±SEM values on the right are the results of four separate experiments.

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**Fig 7.** Ethidium bromide-stained RNA gels. RNA was electrophoresed in 1% agarose as described in "Materials and Methods" and stained with 0.1% ethidium bromide. Gels were photographed on high-speed No. 667 Polaroid film without destaining. Gels were stained after blotting to ensure complete transfer of RNA to the filters (not shown). DOG indicates deoxyglucose; CN, potassium cyanide; and IAA, iodoacetic acid.
with decreased transcript levels of the housekeeping β-actin gene (Figs 4, left, and 6, right). Differences between the KCN-deoxyglucose combination and IAA were probably related to the relative severity of the imposed stress as indicated by cell damage. The KCN-deoxyglucose combination mediated progressive and strong inductions of c-fos and c-jun (fourfold) but did not have any major effects (less than twofold) on the other transcripts. The combination of KCN and deoxyglucose did not impact cell viability within the experimental time, as determined by arachidonic acid or CPK leakage (Fig 5, middle and bottom). In contrast, IAA treatment caused rapid, irreversible, and extensive cell damage that was determined biochemically (Fig 5, middle and bottom) as well as microscopically. Significant induction of all except jun D transcripts occurred simultaneously with cell structural deterioration. IAA treatment was much more toxic than the KCN-deoxyglucose combination. This may be more related to its strong alkylating properties than to metabolic inhibition per se.45,46

Other studies have used metabolic inhibitors, including KCN and IAA, to model ischemic stress.5,46,52-54 In most cases, ATP depletion, probably through the inhibition of ion pumps, caused substantial elevation of intracellular calcium in association with membrane disintegration and irreversible cell damage.46 Therefore, immediate-early gene induction in response to severe metabolic stress involving ATP depletion could be regulated by calcium-dependent pathways. In the case of IAA, the mobilization of lipid second messengers may additionally be involved. Induction of the heat shock protein HSP 70 by hypoxia has also been demonstrated in muscle cells under conditions that involved and were probably related to ATP depletion.55

To date, the molecular signaling mechanisms that regulate most other major hypoxia responsive genes have not been identified; these include erythropoietin,40,56 heme-oxygenase,57 glycolytic enzymes,21,43,48,59 platelet-derived growth factor B-chain,60 endothelin-1,61,62 and a series of oxygen responsive proteins that are detected by two-dimensional gel electrophoresis.63 Interestingly, positive regulation of a tyrosine kinase26 and increased binding of a homeobox transcription factor65 by increased oxidation have recently been described. These findings demonstrate that key cysteine residues in responsive proteins can mediate both positive and negative regulation by redox.

The major conclusion to be drawn from our results is that metabolic inhibition with KCN, at the concentration used in the present study, closely mimicked the effects of hypoxia on metabolic switching but did not reproduce the effects of hypoxia on the expression of immediate-early genes. Compared with hypoxia, KCN mediated inductions that were attenuated both in their magnitude and persistence. Therefore, factors in addition to metabolic switching, possibly related to cellular redox reactions, must be involved in the regulation of these genes by hypoxia.

Acknowledgments

This study was supported by grants from the National Institutes of Health (HL-44578) and the Cigarette and Tobacco Surtax of the State of California through the Tobacco-Related Disease Research Program of the University of California (IR&T-402). We would like to thank Barbara Sato for excellent technical assistance.

I would like to dedicate this article to the memory of my father, Arthur Webster (1913-1994) (K.A.W.).

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doi: 10.1161/01.RES.74.4.679

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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