J Appl Physiol 92: 2200–2207, 2002.
First published December 21, 2001; 10.1152/japplphysiol.01035.2001.

highlighted topics

Molecular Biology of Thermoregulation
Selected Contribution: Hypothermic protection of the ischemic heart via alterations in apoptotic pathways as assessed by gene array analysis

XUE-HAN NING, SHI-HAN CHEN, CHENG-SU XU, LINHENG LI, LENA Y. YAO, KUN QIAN, JULIA J. KRUEGER, OUTI M. HYYTI, AND MICHAEL A. PORTMAN
Divisions of Cardiology and Genetics and Development, Department of Pediatrics, University of Washington, Seattle 98195; and Children's Hospital and Regional Medical Center, Seattle, Washington 98105

Received 11 October 2001; accepted in final form 5 December 2001

Ning, Xue-Han, Shi-Han Chen, Cheng-Su Xu, Linheng Li, Lena Y. Yao, Kun Qian, Julia J. Krueger, Outi M. Hyyti, and Michael A. Portman. Selected Contribution: Hypothermic protection of the ischemic heart via alterations in apoptotic pathways as assessed by gene array analysis. *J Appl Physiol* 92: 2200–2207, 2002. First published December 21, 2001; 10.1152/japplphysiol.01035.2001.—Hypothermia improves resistance to ischemia in the cardioplegia-arrested heart. This adaptive process produces changes in specific signaling pathways for mitochondrial proteins and heat-shock response. To further test for hypothermic modulation of other signaling pathways such as apoptosis, we used various molecular techniques, including cDNA arrays. Isolated rabbit hearts were perfused and exposed to ischemic cardioplegic arrest for 2 h at 34°C [ischemic group (I); $n = 13$] or at 30°C before and during ischemia [hypothermic group (H); $n = 12$]. Developed pressure, the maximum first derivative of left ventricular pressure, oxygen consumption, and pressure-rate product ($P < 0.05$) recovery were superior in H compared with in I during reperfusion. mRNA expression for the mitochondrial proteins, adenine translocase and the β-subunit of F_1-ATPase, was preserved by hypothermia. cDNA arrays revealed that ischemia altered expression of 13 genes. Hypothermia modified this response to ischemia for eight genes, six related to apoptosis. A marked, near fivefold increase in transformation-related protein 53 in I was virtually abrogated in H. Hypothermia also increased expression for the anti-apoptotic Bcl-2 homologue *Bcl-x* relative to I but decreased expression for the proapoptotic Bcl-2 homologue *bak*. These data imply that hypothermia modifies signaling pathways for apoptosis and suggest possible mechanisms for hypothermia-induced myocardial protection.

β-subunit of F_1-ATPase; hypothermic adaptation; myocardial reperfusion

MODERATE HYPOTHERMIA IMPROVES resistance to oxidative injury in the heart. Hypothermic protection can be proffered by application of cold during ischemic insult. In addition, cold stress applied before warm ischemia initiates adaptive responses and promotes preservation of cardiac contractile function (24). This cross-adaptive phenomenon might represent thermotolerance transfer. Hypothermic adaptation is also linked to reduced ATP store depletion during ischemia and reperfusion (27, 28) and is further characterized by enhanced postischemic gene expression for specific stress-related proteins and constitutive mitochondrial membrane proteins (28, 29).

Although modulation of energy metabolism contributes to protection afforded by hypothermia, other injury-reducing mechanisms might also operate in response to cold. Elucidation of these mechanisms could lead to identification of specific molecules that provide protection during and after ischemic insult. Recent experiments in noncardiac tissues indicate that cold modifies various cell signaling pathways. Specifically, investigations performed with cultured neuronal tissues, or in brain in vivo, indicate that mild or moderate hypothermia mitigates apoptosis, programmed cell death induced by hypoxia or ischemia (5, 22, 31). In addition, several studies demonstrate that cardiomyocyte apoptosis contributes to ischemia-reperfusion injury (3, 4, 10, 13, 32). Adaptive processes such as ischemic preconditioning are found to ameliorate reperfusion injury partially through modifications of specific cell death signaling pathways (21). These findings formed the basis for a hypothesis stating that

Address for reprint requests and other correspondence: X.-H. Ning, Dept. of Pediatrics, Box 356320, Univ. of Washington, 1959 NE Pacific St., Seattle, WA 98195 (E-mail: xh@u.washington.edu).

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

hypothermia similarly induces myocardial protection by affecting the signaling for apoptosis and/or other pathways after ischemia and reperfusion. Testing this hypothesis was the principal objective of this study.

The availability of cDNA arrays provides an objective means to examine the molecular profile for multiple genes and identification of changes in signaling pathways in a heart altered by ischemia and hypothermia. This technique and the confirmatory methods, including Northern blot and reverse transcriptive-polymerase chain reaction (RT-PCR), enable testing of the principal hypothesis. Changes in expression of specific genes were associated with functional improvement in cardiac function induced by hypothermia.

MATERIALS AND METHODS

Preparation of isolated heart. Thirty rabbits (male or female, 2.3–2.8 kg body wt) were anesthetized with pentobarbital sodium (45 mg/kg iv) and heparinized (700 U/kg iv). Each heart was rapidly excised and immersed momentarily in ice-cold physiological salt solution, pH 7.4, containing (in mmol/l) 118.0 NaCl, 4.0 KCl, 22.3 $NaHCO_3$, 11.1 glucose, 0.66 KH_2PO_4, 1.23 $MgCl_2$, and 2.38 $CaCl_2$. The aorta was cannulated in the Langendorff mode, and the heart was perfused with physiological salt solution that had been equilibrated with 95% O_2-5% CO_2 at 37°C and passed twice through filters with 3.0-μm pore size. Perfusion pressure was maintained at 90 mmHg (23). An incision was made in the left atrium, and a fluid-filled latex balloon was passed through the mitral orifice and placed in the left ventricle. The fluid from the Thebesian vein did not accumulate in this preparation. The balloon was connected to a pressure transducer for continuous measurement of left ventricular pressure (LVP) and measurement of the first derivative with respect to time. The caudal vena cavum, the left and right cranial vena cava, and the azygous vein were ligated. The pulmonary artery was cannulated to enable collection of coronary flow, measured with a flow meter (T201, Transonic Systems, Ithaca, NY).

Analog signals were continuously recorded on an online computer (Macintosh, Biopac analog signal acquisition system) and a pressurized ink chart recorder (Gould, Cleveland, OH). To characterize cardiac function, we defined developed pressure (DP) as peak systolic pressure minus end-diastolic pressure. Calculating the product of heart rate and DP (PRP) provided an estimate of myocardial work (mmHg/min). Myocardial oxygen consumption ($M\dot{V}O_2$) was calculated as $M\dot{V}O_2 = CF \times [(Pa_{O_2} - Pv_{O_2}) \times (c/760)]$, where CF is the coronary flow ($ml \cdot min^{-1} \cdot g$ wet $tissue^{-1}$), $Pa_{O_2} - Pv_{O_2}$ is the difference in the PO_2 (Torr) between perfusate (arterial) and coronary effluent (venous), and c is the Bunsen solubility coefficient of O_2 in perfusate at 37°C (22.7 μl $O_2 \times atm^{-1} \times ml^{-1}$) (26, 28). Oxygen extraction was calculated as $M\dot{V}O_2$ divided by oxygen content in the perfusate. The wet weight of the heart was determined at the conclusion of each experiment after the fat and great vessels were trimmed and then the heart being blot dried with nine-layer cotton gauze. Procedures followed were in accordance with institutional and National Institutes of Health guidelines.

Lactate, pH, and CO_2 measurements. The coronary inflow and effluent were collected; concentrations of O_2 and CO_2 were immediately measured with a Radiometer (ABL 5, Copenhagen, Denmark). The difference in CO_2 content between the coronary effluent and inflow was calculated as $(Pv_{CO_2} - Pa_{CO_2}) \times c/Vm$, where $Pv_{CO_2} - Pa_{CO_2}$ is the difference in the PCO_2 (Torr) between coronary effluent (venous) and perfusate (arterial), c is the solubility coefficient of CO_2 in perfusate at 37°C (0.53 ml $CO_2 \times atm^{-1} \times ml^{-1}$ perfusate), and Vm is the molar volume ($22.4 \times mM^{-1}$) (26, 28). Lactate concentration was measured with a GM7 analyzer (Analox Micro-Stat, London, UK).

RNA isolation. After removal of excess fat and connective tissues, the left ventricular wall was briefly blotted on nine-layer gauze, frozen in liquid nitrogen, and then stored at −80°C. An aliquot of the frozen heart tissue (100–200 mg) was pulverized and homogenized, and the total RNA was then extracted with a RNA isolation kit (Ambion, Austin, TX). RNA samples were tested by ultraviolet absorption at 260 nm to determine the concentration. The quality and concentration of the RNA samples were further confirmed by electrophoresis on denatured 1% agarose gels (28, 30).

cDNA arrays. The array filters were obtained from Clontech Laboratories (Palo Alto, CA) and Super Array (Bethesda, MD). The Clontech filters had 234 known gene fragments, representing a wide range of genome functions. The Super Array filters contained 68 cDNA gene fragments, immobilized and duplicated on a nylon membrane with specific pathway functions, including transcription signals, stress-inducible apoptosis, DNA repair functions, and mitogenic signal pathways. Procedure was followed as recommended by the companies with minor modifications.

For each experiment, 20 μg of total RNA or 1 μg of poly(A) RNA was used for first-strand synthesized cDNA (SuperScript first-strand synthesis system for RT-PCR, GIBCO BRL, Life Technologies, Gaithersburg, MD). The RNA sample was mixed with 2 μl of 10× cDNA synthesis primer mix and then added to distilled H_2O to a final volume of 6 μl. Incubated tubes were placed at 70°C for 2 min to reduce the temperature of the thermal cycler to 50°C. Fourteen microliters of "master mix" were added, which contained 2× dNTP, 5 mM DTT, [α-^{32}P]dATP (3,000 Ci/mmol), and 50 units of Moloney murine leukemia virus reverse transcriptase. Tubes were incubated at 50°C for 25 min. The reaction was then stopped by adding 1 μl of 10× termination mix. The [^{32}P]cDNA was purified from an unincorporated [^{32}P]dATP by a column chromatography method. The labeled cDNA probe was hybridized overnight in each filter at $\sim 1 \times 10^6$ $counts \cdot min^{-1} \cdot ml^{-1}$. After filters were washed, they were exposed on a PhosphorImager (model 400S, Molecular Dynamics, Sunnyvale, CA) and on Kodak BioMax film (Eastman Kodak, Rochester, NY) at −70°C for various amounts of time.

Specific array signal spots were analyzed with ImageQuant quantitation software (Molecular Dynamics). The relative levels of cDNA expression were assessed by comparing the same signals obtained in normal control, ischemic, and hypothermic conditions. Equivalent amounts of total RNA from five individual and randomly selected heart samples within each experimental group were combined to form subgroups. Instead of testing according to individual sample, the samples were tested according to subgroup to diminish systemic error. The subgroup samples were pooled on each array and repeated four to five times in different membranes. The membranes were stripped and reused two to three times. Relevant changes in gene expression were identified by using decision tree classification (6) and linear discriminant analysis (2). These methods have been adapted for use in arrays by the Fred Hutchison Cancer Research Institute.

RT-PCR analysis. Five micrograms of total RNA were added in 20 μl of reaction mixture and preheated for 10 min at 65°C with 100 ng of poly$(dT)_{12-18}$ primer. The first-strand cDNA was synthesized by SuperScript II RNase H-RT at

42°C for 50 min (Life Technologies). The reaction was terminated at 70°C after 15 min. For subsequent PCR reaction (50 μl), 1 μl of the cDNA mixture was used for each gene-specific amplification. The gene-specific primers for p53 and β-actin were designed by a primer design program (Primer Design 3, Scientific and Educational Software, State Line, PA).

p53 Primers were as follows: 5′-ACCTTCCGACACAGCGTGGT-3′ (p53 forward) and 5′-CTCCATCCAGCGGCTTCTTC-3′ (p53 reversed). β-Actin primers were as follows: 5′-CGAGCGGGAAATCGTGCGTGACATTAAGGAGA-3′ (β-actin$_{478}$ forward), 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′ (β-actin$_{478}$ reversed), 5′-AAAGACCTGTACGCCAACACAGTGCTGTCTGG-3′ (β-actin$_{229}$ forward), and 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′ (β-actin$_{229}$ reversed).

Amplification reactions were conducted in 25–50 μl of reagent mixture with an initial step of 94°C for 3 min followed by 25–35 cycles of amplification, depending on cDNA abundance in preparations. Each cycle was at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and finally at 72°C for 7 min. The β-actin was used as a reference control.

PCR products were analyzed by agarose gel electrophoresis. PCR bands of the predicted size were isolated and subjected to sequencing (ABI Prism 310, Perkin-Elmer Foster) to confirm correct gene identities. Expression levels of specific genes were quantified by Image Station 440cf (Eastman Kodak) on ethidium-stained bands of amplified fragments after normalization to β-actin.

Northern blot analysis. For Northern blot analysis, 15 μg of RNA were denatured and electrophoresed in a 1% formaldehyde agarose gel, transferred to a nitrocellulose transfer membrane (Micron Separations, Westboro, MA), and cross-linked to the membrane with a short-wave ultraviolet cross-linker. The prehybridizing and hybridizing solutions contained 50% formamide, 1× Denhardt's solution, 6× SSPE, and 1% SDS. cDNA probes were labeled with [^{32}P]dCTP by random primer extension (PRIME-IT II, Stratagene, La Jolla, CA) and added to the hybridizing solution to a specific activity. Hybridization was carried out at 42°C for 18 h. The blots were then washed several times with a final wash in 1× standard sodium citrate and 0.1% SDS at 65°C. The blots were exposed through a PhosphorImager (model 400S) and/or on Eastman Kodak BioMax film at −70°C. The relative amounts of mRNA were measured by using ImageQuant quantitation software (Molecular Dynamics). The same size area at each band was taken to measure the intensity, and the same size area at the closest upstream position of each band was used as the background of the image. RNA loading was normalized by comparison to that of 28S and/or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (28, 30). mRNA levels of the β-subunit of F_1-ATPase (βF_1-ATPase) were detected by using a 1.8-kb cDNA fragment cloned from the human HeLa cell line (American Type Culture Collection, Manassas, VA) (28, 30). Adenine nucleotide translocator isoform 1 (ANT_1) mRNA levels were detected by using a 1.4-kb cDNA fragment cloned from human skeletal muscle (American Type Culture Collection) (28, 30). To compare different mRNA levels in the same myocardial sample, 15-μg aliquots of total RNA from the myocardium were analyzed by means of sequentially reprobing the membranes with GAPDH carriers (Clontech), βF_1-ATPase, and ANT_1 cDNA probes.

Experimental protocols. The experimental model used in these experiments has been previously described in detail (26, 28). After we completed instrumentation and performed calibrations, left ventricular balloon volumes were varied over a range of values to construct left ventricular function curves. In this manner, it is possible to define a specific balloon volume that is associated with a DP between 100 and 140 mmHg. This volume remained unchanged during baseline, ischemia, and reperfusion conditions. The intraventricular balloon volumes were not adjusted to produce specific end-diastolic pressures; rather, we defined a level of systolic pressure development. However, end-diastolic pressures at baseline >10 mmHg were not accepted (23). Excluded were data from hearts with DPs <100 mmHg or >140 mmHg. Baseline data were obtained after a 30-min equilibration period. The same procedures were followed in each experiment. During the baseline period, data were obtained with the hearts maintained at 37°C with a temperature-controlled organ bath.

Figure 1 summarizes the hypothermia protocol. To adjust the infused temperature, the myocardial and pulmonary outflow temperatures were monitored continuously with thermal probes. The rabbits were divided into an ischemic group (at 34°C ischemia, $n = 13$) and a hypothermic group (at 30°C, $n = 12$). The hypothermic group was treated with hypothermia before and during ischemia. Hemodynamic data were recorded for 45 min during reperfusion, followed by immersion of the tissue in liquid nitrogen for the Northern blot analysis.

Myocardial temperatures were not measured routinely in each experiment to avoid potential problems associated with traumatic introduction of needle-mounted temperature probes. In parallel experiments, myocardial temperature was monitored with a Khuri regional tissue temperature monitor (Vascular Technology, Chelmsford, MA) (28) to determine changes in the myocardial temperature profile with use of our standard experimental protocol. The results showed the pulmonary outflow temperature could represent myocardial temperature. Myocardial samples were also taken from five hearts in situ as a nonperfused normal control group (N; $n = 5$), for molecular comparisons.

Statistical analysis. The values are reported as mean ± SE. The Statview 4.5 (FPV) program (Abacus Concepts, Berkeley, CA) was used for statistical analysis. Data were

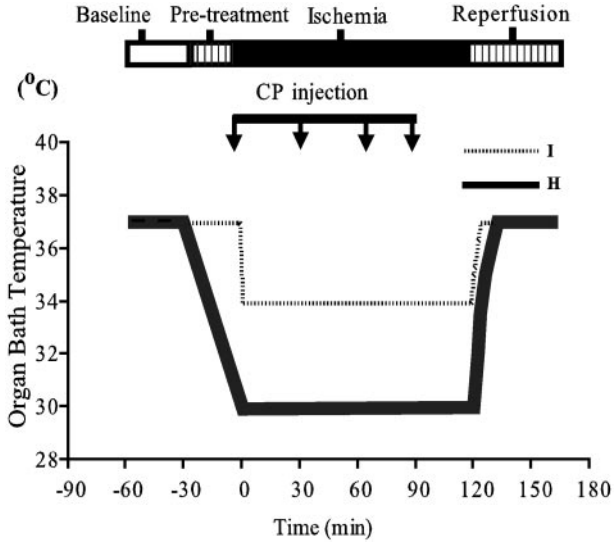

Fig. 1. Protocol of hypothermia in ischemic hearts. I, ischemic group; H, hypothermic group (hypothermia treatment before and during ischemia); CP, cardioplegic solution. Ischemia starts at 0 min and ends at 120 min and then reperfusion follows. There are no significant differences in temperature among the groups during baseline and reperfusion.

Table 1. *Hemodynamics*

	Baseline	Reperfusion 15 min	Reperfusion 30 min	Reperfusion 45 min
EDP, mmHg				
I	1.9 ± 0.3	39.2 ± 4.3	33.3 ± 4.3	30.9 ± 4.2
H	2.6 ± 0.4	5.1 ± 0.9*	5.2 ± 1.2*	6.2 ± 1.6*
DP, mmHg				
I	119.9 ± 3.2	27.5 ± 3.8	40.5 ± 5.0	43.5 ± 5.3
H	120.6 ± 2.7	104.7 ± 2.7*	108.6 ± 3.6*	107.0 ± 4.3*
dP/dt_{max}, mmHg/s				
I	1821 ± 136	360 ± 45	524 ± 67	581 ± 77
H	1715 ± 53	1555 ± 84*	1660 ± 111*	1631 ± 112*
$-dP/dt_{max}$, mmHg/s				
I	1254 ± 108	307 ± 37	407 ± 48	463 ± 53
H	1377 ± 68	1249 ± 56*	822 ± 120*	1264 ± 55*
HR, beats/min				
I	185.2 ± 8.5	169.7 ± 16.4	165.3 ± 12.8	167.3 ± 12.3
H	187.0 ± 7.6	183.3 ± 9.6	184.1 ± 7.8	186.8 ± 7.2
PRP, 10^3 mmHg/min				
I	22.25 ± 1.26	4.55 ± 0.68	6.41 ± 0.87	6.90 ± 0.85
H	22.48 ± 0.91	19.25 ± 1.20*	20.02 ± 1.12*	20.01 ± 1.16*
$M\dot{V}O_2$, $\mu l \cdot min^{-1} \cdot g^{-1}$				
I	99.2 ± 5.4	41.5 ± 3.9	39.8 ± 4.4	44.5 ± 5.1
H	111.7 ± 8.2	95.3 ± 5.9*	99.6 ± 4.2*	100.6 ± 5.1*

Values are means ± SE. The hemodynamic indexes were determined in isolated reperfused hearts at baseline and at 15, 30, and 45 min of reperfusion as described in MATERIALS AND METHODS. I, ischemic control group (n = 13); H, hypothermia treatment of combining before and during ischemia (n = 12); DP, developed pressure; $\pm dP/dt_{max}$, maximum of the positive or negative first derivative of left ventricular pressure with respect to time; EDP, end-diastolic pressure; HR, heart rate; PRP, product of HR and DP; $M\dot{V}O_2$, myocardial oxygen consumption. *$P < 0.05$, compared with I.

evaluated with repeated-measures ANOVA within groups and with single-factor ANOVA across groups. When significant data were obtained with Scheffé's F test, individual group means were tested for differences with F values (two-tailed test). The criterion for significance was $P < 0.05$ for all comparisons (30).

RESULTS

Functional recovery during reperfusion. The heart weight and left ventricular balloon volume were similar in the ischemic (6.80 ± 0.45 g and 1.60 ± 0.045 ml, respectively) and the hypothermic treatment groups (6.15 ± 0.25 g and 1.46 ± 0.034 ml, respectively). Under the baseline conditions, there appeared to be no significant difference between the groups in end-diastolic pressure, DP, maximum of the positive or negative first derivative of LVP with respect to time, heart rate, PRP, and $M\dot{V}O_2$. Hemodynamic results are summarized in Table 1 and Fig. 2. The hypothermia-treated hearts demonstrated superior functional recovery compared with the ischemic group.

Ischemic contracture. As noted in MATERIALS AND METHODS, we used a specific balloon volume that was adjusted and maintained throughout the protocol. This allowed comparisons of LVP under constant end-diastolic volume. After we injected the cardioplegic solution, the LVP was always near 0 mmHg. We defined the beginning of ischemic contracture by the initial rise in LVP above 2 mmHg. Ischemic contracture began in control hearts after 69.8 ± 4.4 min of ischemia. No ischemic contracture was observed after 120 min of ischemia in the hypothermia-treated hearts.

Accumulation of catabolic products. An obvious increase in accumulation of lactate and CO_2 was noted in ischemic hearts at 120 min of ischemia (Fig. 3). Accumulation of both metabolites was significantly lower in hypothermic hearts. These data imply that aerobic and anaerobic metabolic rates were markedly decreased with hypothermia treatment.

Gene expression for mitochondrial-specific proteins. Northern blot analysis showed that βF_1-ATPase and ANT_1 mRNA were preserved by hypothermic treat-

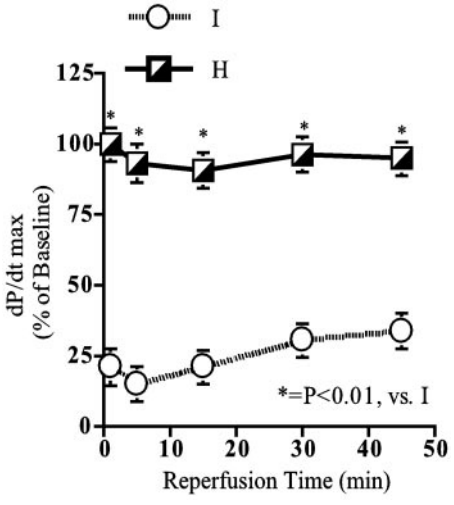

Fig. 2. Effect of hypothermia on functional recovery during reperfusion. The cardiac function parameters are plotted as a percent of baseline. dP/dt_{max}, the first derivative of left ventricular pressure with respect to time (n = 13 in I; n = 12 in H).

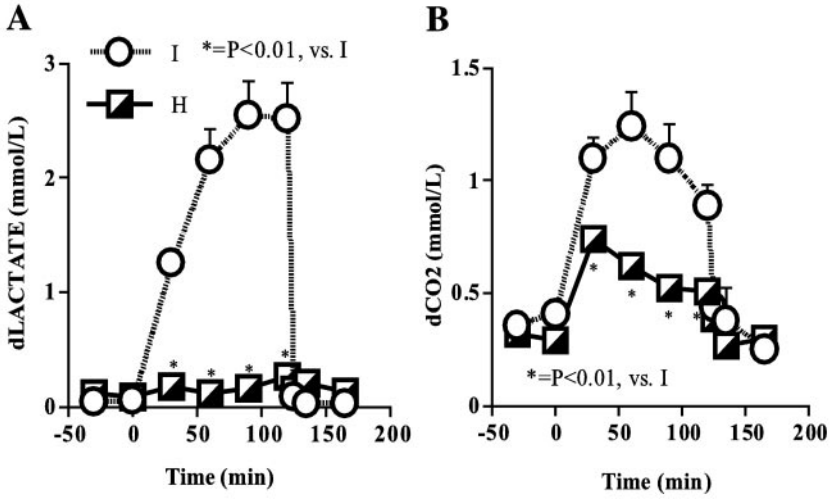

Fig. 3. Metabolites during hypoxia. *A*: dLACTATE (difference in lactate content between the coronary outflow and inflow). *B*: dCO_2 (difference in CO_2 content between the coronary outflow and inflow). Ischemia starts at 0 min and ends at 120 min and then reperfusion follows. There is no significant accumulation in dLACTATE during ischemia compared with baseline and reperfusion in H. dCO_2 is significantly lower in H ($n = 12$) than in I ($n = 13$).

ment. The intensity of βF_1-ATPase and ANT_1 mRNA were normalized to GAPDH. mRNA βF_1-ATPase levels were 3.0 ± 0.4 greater in the hypothermic than in the ischemic group ($P < 0.01$). mRNA ANT_1 levels were 2.9 ± 0.3 greater in the hypothermic than in the ischemic group ($P < 0.01$) (Fig. 4).

cDNA array analysis. cDNA arrays were used to determine the abundance of expressed genes in hearts from the normal control, ischemic, and hypothermic-ischemic groups (Fig. 1). The arrays consisted of the following functional gene groups: *1*) stress response regulator and effector; *2*) apoptosis, DNA synthesis, damage repair, and replication; *3*) drug metabolism; *4*) mitogenic signal pathways; *5*) other signal pathways; *6*) transcription factors and DNA binding proteins; and *7*) receptors, cell-surface antigens, and adhesions. cDNAs of β-actin and GAPDH served as controls.

We identified 40 hybridization signals of the 302 (13.2%) genes on the arrays after 12-h to 8-day exposures. Only 13 of the 40 genes (Table 2) showed significant change in the ratio of adjusted intensity, that is, >2 or <0.5 related to the control or to the ischemic group. Ten genes with a significant increase in hybridization signal after 45 min of ischemia treatment were categorized, including early growth response 1 (*egr-1*), growth arrest and DNA damage-inducible gene (*Gadd45* and *Gadd45β*), cyclin-dependent kinase inhibitor 1A ($p21^{waf1}$), and transformation-related p53, as well as others. Seven of the 10 genes belonged to the apoptosis pathways (p53 signal pathway). Hypothermic treatment decreased the hybridization signal in 5 of the 10 genes. There was a 4.7-fold increase in p53 expression in the ischemic group compared with the control group; hypothermia reversed it to 0.15-fold of that shown for the ischemic group (Table 2, Fig. 5). The hybridization signal increased for three genes during hypothermic treatment, including a gene of anti-apoptotic Bcl-2 homologue (*Bcl-x*), whereas the proapoptotic Bcl-2 homologue, *bak*, exhibited a significant decrease in expression.

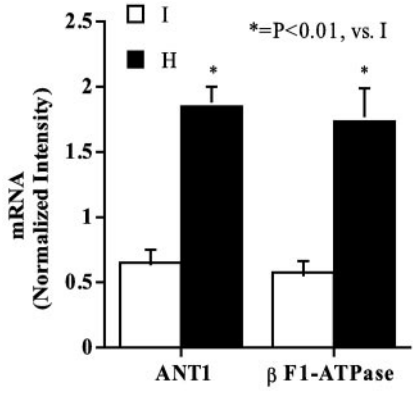

Fig. 4. Steady-state mRNA levels of β-subunit of F_1-ATPase (βF_1-ATPase) and adenine nucleotide translocator isoform 1 (ANT_1). Transcript levels are relative to 28S band intensity and to the normal control heart ($n = 5$) in situ (N).

RT-PCR analysis. Changes in p53 expression were further confirmed by RT-PCR amplifications of the control, ischemic, and hypothermic groups (Fig. 6). Correct identification of the p53 sequence was first verified by DNA sequencing (results not shown). The relative intensity of p53 expression in the ischemic group compared with normal was greater than fourfold. Expression in the hypothermic group displayed expression 0.3 times that of the ischemic group ($P < 0.05$). Thus the RT-PCR results correspond to the findings of cDNA arrays.

DISCUSSION

Hypothermia ameliorates oxidative injury induced during myocardial ischemia and reperfusion. Accordingly, cardiac function during reperfusion in this study was improved by application of hypothermia beginning before and extending through the ischemic period. Hypothermic protection is generally attributed to elevations in the energy supply-to-demand ratio during ischemia. This has been previously substantiated in ischemic cardioplegia-arrested rabbit heart by superior

Table 2. *Hypothermia effect on gene expression*

Pathway	Gene	I	H	N	H/I	I/N
Housekeeping	GAPDH*	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.07	1.00	1.00
Mitogenic signal	Early growth response 1 (*egr-1*)	1.12 ± 0.01	1.17 ± 0.01	0.15 ± 0.03	1.04	7.47
CREB signal	Cytochrome *P*-450, 19, aromatase	0.01 ± 0.01	0.23 ± 0.04		23.0	
Aerobic pathway	Succinodehydrogenase	1.39 ± 0.03	0.07 ± 0.01	0.70 ± 0.01	0.05	1.99
Stress signal	PPAR-γ coactivator 1 (PGC1)	1.67 ± 0.01	1.41 ± 0.01	0.81 ± 0.01	0.84	2.06
	Hypoxia-inducible factor-1α (HIF-1α)	0.13 ± 0.01	0.91 ± 0.01	0.13 ± 0.01	7.00	1.00
Apoptosis	Caspase10	0.11 ± 0.003	0.07 ± 0.01	0.01 ± 0.01	0.64	11.0
	DNA damage-inducible transcript 1 (*Gadd45*)	0.53 ± 0.01	0.19 ± 0.02	0.10 ± 0.01	0.36	5.30
	*Gadd45*β	0.10 ± 0.01	0.06 ± 0.01	0.03 ± 0.02	0.60	3.33
	Cyclin-dependent kinase inhibitor 1A (*p21*)	0.65 ± 0.01	0.75 ± 0.02	0.20 ± 0.04	1.15	3.25
	IκBa (*mad3*)	0.14 ± 0.02	0.05 ± 0.01	0.03 ± 0.02	0.36	4.67
	Bc12-antagonist/killer 1 (*bak*)	0.65 ± 0.04	0.32 ± 0.01		0.49	
	Bc12-like 1 (*bcl-x*)	0.87 ± 0.07	2.67 ± 0.03		3.07	
	Transformation-related protein 53 (p53)	0.84 ± 0.05	0.13 ± 0.01	0.18 ± 0.02	0.15	4.67

Values are means ± SE. *Gene intensities are normalized by the average value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same membrane. H/I, ratio of mean values of hypothermia treatment vs. ischemia; I/N, ratio of mean values of ischemia vs. normal control. The expression of some genes could not be detected in the normal control.

ATP preservation induced by hypothermia applied either before (28) or during ischemia (29). High-energy phosphate store preservation is subject to a critical temperature threshold (30°C), above which this advantage rapidly dissipates (27). The current experiments employed hypothermic temperatures both before and during ischemia at 30°C. This protocol thus provides adaptation, characteristic of cold tolerance, and resistance to injury during the oxidative insult. Complementing these two protective mechanisms of hypothermia optimizes metabolic, functional, and gene expression differences between this experimental group and the ischemic group. Elevations in lactate and CO_2 production throughout ischemia occurred in the normothermic group, thus confirming increased energy utilization compared with the hypothermic hearts throughout the ischemic period (27, 28).

Previous studies in this laboratory indicate that hypothermia applied either before or during ischemia promotes gene expression for the constitutive mitochondrial membrane proteins: ANT and βF_1-ATPase. This phenomenon, confirmed in the present study, occurs along with accelerated induction of gene expression for heat shock protein 70 (26, 28, 29) and emulates the induction of these genes by cold stress in cold-adapted tissues (14). The previous investigations addressed expression for these specific genes and did not evaluate other pathways that could be altered by hypothermia. Subsequently, several investigators noted participation of the ANT in formation of the permeability transition pore complex (PTPC) within the inner mitochondrial membrane. This complex presumably promotes leakage of protons and ions from the mitochondrial matrix and leads to loss of mitochondrial integrity, initiating sequence activation of apoptotic pathways. ANT protein expression tightly coordinates with steady-state mRNA levels in several animal models. Thus one might presume that the elevated ANT gene expression in the hypothermic hearts after reperfusion heralds accelerated ANT synthesis and accumulation in the mitochondrial membrane. This process could lend stability to the mitochondrial membrane and thwart mechanisms leading to apoptosis, now a recognized mechanism of cardiomyocyte death following oxidative injury (3, 4, 10, 11, 13, 19). These speculations led to the consideration that hypothermia could alter other pathways related to mitochondrial membrane integrity and stability.

Expression patterns for various genes documented in this study support the validity of this hypothesis. A principal finding in this study was that a relatively mild degree of hypothermia modified postischemic gene expression for several proteins that contribute to the regulation of apoptosis. Hypothermia blunted postis-

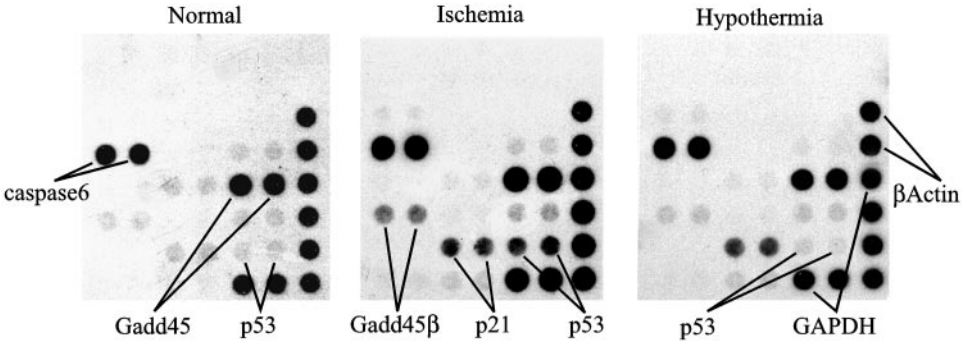

Fig. 5. A representative cDNA array for p53. The intensities of p53 are much higher in I than in N and H, but the intensities of β-actin are the same in the 3 groups.

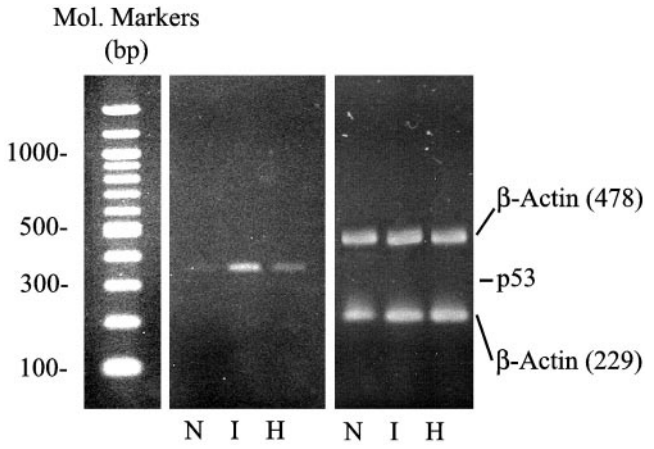

Fig. 6. RT-PCR of a p53 fragment (349 bp) after 35 cycles of amplification. Densitometry analysis of average ratio of p53 are 4.0 (I/N) and 0.3 (H/I) ($P < 0.05$); however, there are no significant difference in the ratio of β-actin between the groups (I/N, H/I, $P > 0.5$). Mol., molecular.

chemic expression for the transformation-related p53 (tumor suppressor p53) as well as for various genes that are considered its targets. Previous studies have linked p53 protein expression with the morphological changes and genomic DNA fragmentation characteristic of apoptosis after oxidative stress in cardiomyocytes (19). The p53 protein serves as a transcriptional activator of a number of target genes, including *Gadd45* (8, 37), *p21*, caspase-3, and others (33). The temperature-induced modifications in p53 response to ischemia in heart have not been previously noted and represent an area for further research.

Changes in expression for two members of the *Gadd45* family during ischemia represent the type of serendipitous results often yielded by cDNA microarray analyses. Hypothermic modification of the *Gadd45* and *Gadd45β* responses to ischemia represents an additional and novel finding generated by cDNA array technique. The *Gadd45* family is expressed in a variety of tissues and is upregulated by hypoxia or stress (10, 13, 33). An extensive literature review yielded only a single previous evaluation of expression for this gene in the heart (34). Specifically, Rees (34) noted *Gadd45* expression decreases in rat heart during fetal development. p53 upregulates *Gadd45*, which inhibits DNA synthesis and allows repair of damaged DNA in specific cell types demonstrating mitotic activity. However, the functions of *Gadd45* and *Gadd45β* have not been examined in cardiomyocytes. Therefore, the implications of changes in expression for the *Gadd45* genes in heart are undetermined.

The increased expression of the *p21* gene after ischemia is consistent with the hypoxic response in cardiomyocytes previously demonstrated by Long and coauthors (19). Although *p21* is a target gene of p53 (8), its expression can also be induced independently by reactive oxygen species (35). Therefore, lack of suppression of the *p21* response by hypothermia might reflect induction by a separate pathway, not regulated by p53 or subject to thermoregulation. In addition, failure of hypothermia to modify response of *p21* expression to ischemia, as well as that of *egr-1*, demonstrates that hypothermia modification is not ubiquitous and might indicate the presence of specific thermally activated cofactors.

Mitochondrial membrane integrity depends on Bcl-2 family expression. Members of the Bcl-2 gene family both positively and negatively regulate apoptosis. Bcl-2 localizes in the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum, and nuclear envelope (1, 16). The Bcl-2 protein prevents the cascade leading from the opening of the PTPC complex to cytochrome *c* release, caspase activation, and cell death. Regulation of apoptosis is highly dependent on the ratio of antiapoptotic to proapoptotic proteins. The proapoptotic Bcl-2 family member *bak*, considered in the present study, alters mitochondrial stability and enhances apoptotic cell death (17), whereas *Bcl-xl* forms antiapoptotic heterodimers with Bcl-2. Our results confirm findings from previous studies by demonstrating that *bak* and *Bcl-x* are not highly expressed in normal heart tissue (7, 20). However, hypothermia markedly accentuates ischemic induction of *Bcl-x* and abrogates *bak* expression. Thus the hypothermia application modifies Bcl-2 family expression in directions that would be expected to stabilize the mitochondrial membrane and prevent apoptosis.

The majority of microarray studies have not reported an analysis of specificity (18). Nor have strategies for identification of differences in array signal intensities been well described. This lack of knowledge could diminish the value of the current data and represent a limitation of this study. However, a recently published analysis of arrays in heart demonstrated that specificity can be increased substantially by performing repetitive experiments. We repeated array experiments at least four to five times to confirm reproducibility of our results. In addition, traditional methods of discrimination were modified to analyze these arrays and limit false-positive results. Finally, RT-PCR confirmed changes in gene expression for p53. RT-PCR results for this gene were virtually identical to results obtained from array analysis.

In summary, through use of cDNA arrays, these experiments identified the induction of several genes by ischemia in heart. In particular, hypothermic modification of p53 and ischemic induction of *Gadd45* and *Gadd45β* are novel findings in cardiac tissue. Furthermore, hypothermic modification of several signaling pathways in directions presumed to promote antiapoptotic factors was documented. These findings, paired with preserved gene expression for constitutive mitochondrial proteins imply that hypothermia promotes signaling for mitochondrial membrane stability after ischemia-reperfusion injury.

We thank Dr. W. G. Guntheroth, Department of Pediatrics, for advice.

This work was funded in part by National Heart, Lung, and Blood Institute Grant HL-60666-1 and Children's Hospital and Regional Medical Center Research Fund Grant HR-5836.

REFERENCES

1. **Akao Y, Otsuki Y, Kataoka S, Ito Y, and Tsujimoto Y.** Multiple subcellular localization of Bcl-2: detection in nuclear outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes. *Cancer Res* 54: 2468–2471, 1994.
2. **Armitage P and Berry G.** *Statistical Methods in Medical Research* (3rd ed.). Cambridge, UK: Blackwell Science, 1995, p. 358–371.
3. **Bartling B, Holtz J, and Darmer D.** Contribution of myocyte apoptosis to myocardial infarction. *Basic Res Cardiol* 93: 71–84, 1998.
4. **Bialik S, Geenen DL, Sasson IE, Cheng R, Horner JW, Evans SM, Lord EM, Koch CJ, and Kitsis RN.** Myocyte apoptosis during acute myocardial infarction in the mouse localizes to hypoxic regions but occurs independently of p53. *J Clin Invest* 100: 1363–1372, 1997.
5. **Bossenmeyer-Pourie C, Koziel V, and Daval JL.** Effects of hypothermia on hypoxia-induced apoptosis in cultured neurons from developing rat forebrain: comparison with preconditioning. *Pediatr Res* 47: 385–391, 2000.
6. **Breiman LEO, Friedman JH, and Stone CJ.** *Classification and Regression Trees*. Pacific Grove, CA: Wadsworth & Brooks, 1985, p. 2–21, 31, 51–54.
7. **Chen F, Lu Y, Zhang Z, Vallyathan V, Ding M, Castranova V, and Shi X.** Opposite effect of NF-κB and c-Jun N-terminal kinase on p53-independent Gadd45 induction by arsenite. *J Biol Chem* 276: 11414–11419, 2001.
8. **El-Deiry WS.** Regulation of p53 downstream genes. *Semin Cancer Biol* 8: 345–357, 1998.
9. **Finkel E.** The mitochondrion: is it central to apoptosis? *Science* 292: 624–626, 2001.
10. **Fliss H.** Accelerated apoptosis in reperfused myocardium: friend of foe? *Basic Res Cardiol* 93: 90–93, 1998.
11. **Gottlieb RA, Burleson KO, Kloner RA, Babior BM, and Engler RL.** Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 94: 1621–1628, 1994.
12. **Jin S, Zhao H, Fan F, Blanck P, Fan W, Colchagie AB, Fornace AJ, and Zhan Q.** BRCA1 activation of the Gadd45 promoter. *Oncogene* 19: 4050–4057, 2000.
13. **Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, and Anversa P.** Apoptotic and necrotic myocyte cell deaths are independent contributing variable of infarct size in rats. *Lab Invest* 74: 86–107, 1996.
14. **Klingenspor M, Ivemeyer M, Wiesinger H, Haas K, Heldmaier G, and Wiesner RJ.** Biogenesis of thermogenic mitochondria in brown adipose tissue of Djungarian hamsters during cold adaptation. *Biochem J* 316: 607–613, 1996.
15. **Koong AC, Denko NC, Hudson KM, Schindler C, Swiersz L, Koch C, Evans S, Ibrahim H, Le QT, Terris DJ, and Giaccia AJ.** Candidate gene for the hypoxic tumor phenotype. *Cancer Res* 60: 883–887, 2000.
16. **Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, and Reed JC.** Investigation of the subcellular distribution of the Bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res* 53: 4701–4714, 1993.
17. **Latif N, Khan MA, Briks E, O'Farrell A, Westbrook J, Dunn MJ, and Yacoub MH.** Upregulation of the Bcl-2 family of proteins in end stage heart failure. *J Am Coll Cardiol* 35: 1769–1777, 2000.
18. **Liu TJ, Lai HC, Wu W, Chinn S, and Wang PH.** Developing a strategy to define the effects of insulin-like growth factor-1 on gene expression profile in cardiomyocytes. *Circ Res* 88: 1231–1238, 2001.
19. **Long X, Boluyt MO, Hipolito ML, Llundberg MS, Zheng JS, O'Neill L, Cirielli C, Lakatta EG, and Crow MT.** p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes. *J Clin Invest* 99: 2635–2643, 1997.
20. **Lyn D, Liu X, Bennett NA, and Emmett NL.** Gene expression profile in mouse myocardium after ischemia. *Physiol Genomics* 2: 93–100, 2000.
21. **Maulik N, Engelman RM, Rousou JA, Flack JE III, Deaton D, and Das DK.** Ischemic preconditioning reduces apoptosis by upregulating anti-death gene Bcl-2. *Circulation* 100: 369–375, 1999.
22. **Mizuno T, Miura-Suzuki T, Yamashita H, and Mori N.** Distinct regulation of brain mitochondrial carrier protein-1 and uncoupling protein-2 genes in the rat brain during cold exposure and aging. *Biochem Biophys Res Commun* 278: 691–697, 2000.
23. **Ning XH, Ding X, Childs KF, Bolling SF, and Gallagher KP.** Flavone improves functional recovery after ischemia in isolated reperfused rabbit hearts. *J Thorac Cardiovasc Surg* 105: 541–549, 1993.
24. **Ning XH, Xu CS, Song YC, Xiao Y, and Portman MA.** Hypothermia preconditioning in isolated rabbit heart (Abstract). *FASEB J* 10: A36, 1996.
25. **Ning XH, Xu CS, Chen SH, Yao Y, Song YC, Li L, Lupinetti FM, and Portman MA.** Application of the "threshold-temperature" (30°C) for ischemic myocardial protection in isolated rabbit hearts (Abstract). *FASEB J* 13: A1064, 1999.
26. **Ning XH, Xu CS, and Portman MA.** Mitochondrial protein and HSP70 signaling after ischemia in hypothermic-adapted hearts augmented with glucose. *Am J Physiol Regulatory Integrative Comp Physiol* 277: R11–R17, 1999.
27. **Ning XH, Xu CS, Song Y, Childs KF, Xiao Y, Bolling SF, Lupinetti FM, and Portman MA.** Temperature threshold and modulation of energy metabolism in the cardioplegic arrested rabbit heart. *Cryobiology* 36: 2–11, 1998.
28. **Ning XH, Xu CS, Song Y, Xiao Y, Hu Y, Lupinetti FM, and Portman MA.** Hypothermia preserves function and signaling for mitochondrial biogenesis during subsequent ischemia in isolated rabbit heart. *Am J Physiol Heart Circ Physiol* 274: H786–H793, 1998.
29. **Ning XH, Xu CS, Song Y, Xiao Y, Hu Y, Lupinetti FM, and Portman MA.** Temperature threshold and preservation of signaling for mitochondrial membrane proteins during ischemia in rabbit heart. *Cryobiology* 36: 321–329, 1998.
30. **Ning XH, Zhang J, Liu J, Ye Y, Chen SH, Form AHL, Bache RJ, and Portman MA.** Signaling and expression for mitochondrial membrane proteins during left ventricular remodeling and contractile failure after myocardial infarction. *J Am Coll Cardiol* 32: 282–287, 2000.
31. **Phanithi PB, Yoshida Y, Santana A, Su M, Kawamura S, and Yasui N.** Mild hypothermia mitigates post-ischemic neuronal death following focal cerebral ischemia in rat brain: immunohistochemical study of Fas, caspase-3 and TUNEL. *Neuropathology* 20: 273–282, 2000.
32. **Piot CA, Padmanaban D, Ursell PC, Sievers RE, and Wolfe CL.** Ischemic preconditioning decreases apoptosis in rat hearts in vivo. *Circulation* 96: 1598–1604, 1997.
33. **Polyak K, Xia Y, Zweiert JL, Kinzler KW, and Vogelstein B.** A model for p53-induced apoptosis. *Nature* 389: 300–305, 1997.
34. **Rees WD, Hay SM, Fontanier-Razzag NC, Antipatis C, and Harries DN.** Expression of the growth arrest genes (GAS and Gadd45) changes during organogenesis in the rat fetus. *J Nutr* 129: 1532–1536, 1999.
35. **Russo T, Zambrano N, Esposito F, Ammendola R, Cimino F, Fiscella M, Jackman J, O'Connor PM, Anderson CW, and Appella E.** A p53-independent pathway for activation of WAF1/CIP1 expression following oxidative stress. *J Biol Chem* 270: 29386–29391, 1995.
36. **Schmidt-Kastner R, Zhao W, Truettner J, Belayev L, Busto R, and Ginsberg MD.** Pixel-based image analysis of HSP70, Gadd45 and MAP2 mRNA expression after focal cerebral ischemia: hemodynamic and histological correlates. *Brain Res Mol Brain Res* 63: 79–97, 1998.
37. **Velasco-Miguel S, Buckbinder L, Jean P, Gelbert L, Talbott R, Laidlaw J, Seizinger B, and Kley N.** PA26, a novel target of the p53 tumor suppressor and member of the Gadd45 family of DNA damage and growth arrest inducible genes. *Oncogene* 18: 127–137, 1999.