Impact of *N*-Acetylcysteine on Neonatal Cardiomyocyte Ischemia-Reperfusion Injury

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ABSTRACT: Reactive oxygen species (ROS) are hypothesized to play a key role in myocardial ischemia-reperfusion (IR) injury after cardiopulmonary bypass in children. Clinical studies in adults and several animal models suggest that myocardial IR injury involves cardiomyocyte apoptosis and necrosis. This study investigated a potential relationship between IR-induced ROS production and neonatal cardiomyocyte apoptosis using both *in vitro* and *ex vivo* techniques. For *in vitro* experiments, embryonic rat cardiomyocytes (H9c2 cells) exposed to hypoxia-reoxygenation (HR) showed a time-dependent increase in gp91^{phox} (a marker for ROS production by NADPH oxidases), caspase-3 (a key mediator of apoptosis) expression, and a decrease in the glutathione redox ratio. *N*-acetylcysteine (NAC; 0.25–2 mM), a potent antioxidant, decreased gp91^{phox} and caspase-3 expression, inhibited apoptosis and restored the glutathione redox ratio. For *ex vivo* study, IR injury significantly reduced left ventricular (LV) function and increased the expression of gp91^{phox} and caspase-3 in Langendorff-perfused neonatal (7–14 d) rabbit hearts. NAC (0.4 mM) treatment completely attenuated LV dysfunction after IR. In summary, neonatal myocardial IR injury is associated with an increase in cardiomyocyte oxidative stress and apoptosis. NAC attenuates apoptosis in an *in vitro* embryonic rat cardiomyocyte model of HR, and myocardial dysfunction in an *ex vivo* neonatal rabbit model of myocardial IR injury. (*Pediatr Res* **70:** 61–66, 2011)

Myocardial ischemia-reperfusion (IR) injury associated with cardiopulmonary bypass and cardioplegic arrest contributes to adverse outcomes after cardiac surgery. The pathogenesis of IR injury is complex, but reactive oxygen species (ROS) generated during IR are thought to play a pivotal role leading to membrane lipid peroxidation, protein denaturation, and DNA modification, all of which may result in irreversible myocyte injury and cell death. Oxygen free radicals are cytotoxic molecules generated during reperfusion and/or reoxygenation of a previously hypoxic tissue bed. The cell damage induced by ROS can also initiate a local inflammatory response, which leads to further oxidant stress-mediated tissue damage (1).

Several studies have suggested that ROS are involved in the pathogenesis of perinatal asphyxia (2). Neonates, especially those born prematurely, are particularly vulnerable to ROS-mediated tissue damage due, in part, to their immature native antioxidant system (3,4). Similarly, myocardial IR injury

seems to be a more clinically significant problem in infants after cardiac surgery compared with adults (5–7). Several ROS-producing systems have been identified in many cell types. NADPH oxidase is reported to be a primary source of ROS production in cardiac tissue, and gp91^{phox} is responsible for the catalytic activity of NADPH oxidase (8,9). Increased ROS induce apoptosis in cardiomyocytes. Caspase-3 protein is a member of the cysteine-aspartic acid protease family that has been identified as being a key mediator of apoptosis in mammalian cells (10,11).

N-acetylcysteine (NAC) is a thiol-containing molecule that acts as a free radical scavenger. In addition, NAC is a glutathione precursor that increases intracellular antioxidant capacity (12). Clinically, NAC is indicated for the treatment of acetaminophen overdose, for the prevention of radiocontrast-induced nephropathy, and as an adjuvant in respiratory conditions with excessive and/or thick mucus production. However, there are contradictory reports on the effects of NAC in clinical (13,14) and animal studies (15–17) of myocardial IR injury. Furthermore, there are limited data specifically focused on NAC use during infancy.

The aim of our study was to evaluate the effects of NAC in neonatal cardiomyocytes exposed to IR. Therefore, we investigated the use of NAC in a cultured embryonic rat cardiomyocyte model of hypoxia-reoxygenation (HR) and in a neonatal rabbit model of IR injury to test the hypothesis that NAC attenuates IR injury associated with ROS-mediated cardiomyocyte apoptosis in neonates.

MATERIALS AND METHODS

Cell line, culture conditions, and treatment. Embryonic rat heart derived H9c2 (2–1) cells (ATCC, Manassas, VA) were cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 10% FBS (fetal bovine serum, GIBCO) and antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin, GIBCO) at 37°C in a 95% room air/5% CO_2 incubator. H9c2 cells were grown to the desired confluence, the growth medium was replaced with glucose-free, serum-free medium containing 2% oxyrase (Oxyrase, Mansfield, OH), and the cells were transferred to a hypoxic chamber. A hypoxic environment (<1% O_2) was achieved using a modular incubator chamber gassed with 95% N_2/5% CO_2. Oxygen concentration was constantly monitored during incubation using a microprocessor-based oxygen sensor. Subsequently, media was replaced with standard culture media during a 2 h period of room air reoxy-

Abbreviations: **AAD,** amino-actinomycin; **GSH,** reduced form of glutathione; **GSSG,** oxidized form of glutathione; **HR,** hypoxia-reoxygenation; **IR,** ischemia-reperfusion; **LV,** left ventricle; **LVDP,** left ventricular developed pressure; **NAC,** *N*-acetylcysteine; **PE,** phycoerythrin; **ROS,** reactive oxygen species
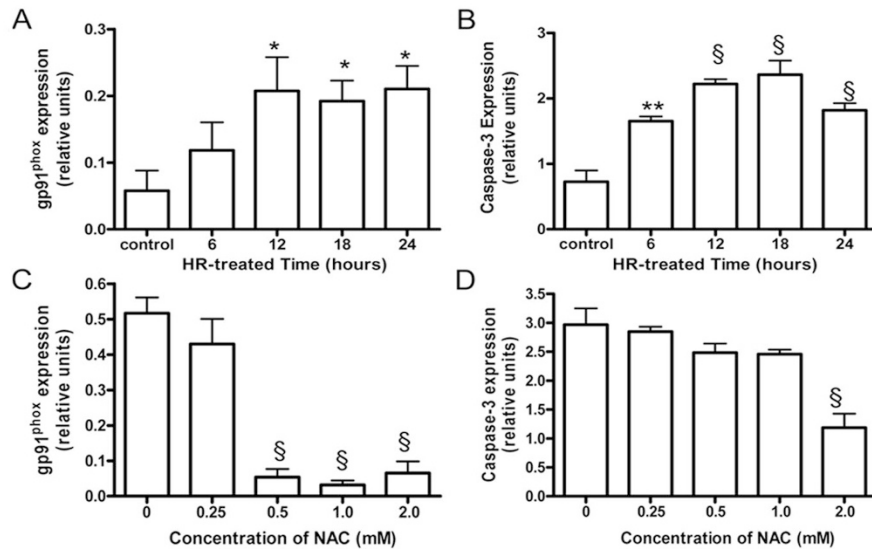
Figure 1. Effect of NAC on protein expression of gp91^{phox} and caspase-3 in HR-induced H9c2 cells. H9c2 cells were exposed to varying lengths of HR and protein levels of gp91^{phox} (*A*) and caspase-3 (*B*) were analyzed by immunoblot analysis. Then H9c2 cells were treated with different concentration of NAC (0.25–2.0 mM) or vehicle for 12 h HR and gp91^{phox} (*C*) and caspase-3 (*D*) protein expression were measured. The results are mean ± SE of five or six different cell cultures. *$p < 0.05$, **$p < 0.01$, §$p < 0.001$ *vs* control.

genation. To investigate the role of ROS, H9c2 cells were treated with different concentration (0.25–2.0 mM) of NAC (Sigma Chemical Co.-Aldrich, St. Louis, MO). Oxidative stress was measured by immunoblotting, glutathione redox levels, and flow cytometry.

Protein extraction and immunoblot analysis. Cells were extracted with RIPA lysis buffer (Pierce, Rockford, IL) and Complete Protease inhibitor cocktail (Roche Diagnostic, Indianapolis, IN). Protein concentrations were determined by the BCA method (Pierce). Cell lysates (30 µg) were separated by 10% (for gp91^{phox}) or 12% (for caspase-3) SDS PAGE, and proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratory, Hercules, CA). The membrane was blocked with 5% nonfat milk and then incubated with specific primary antibody against gp91^{phox} (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and caspase-3 (1:200, Santa Cruz). After being washed, the blots were incubated with a horseradish peroxidase-conjugated appropriate secondary antibody (1:2500, Santa Cruz). Detection was made with enhanced chemiluminescence detection system (ECL) from Pierce. Membranes were also probed with actin (1:200, Santa Cruz) to control for protein loading conditions. Blot images were quantified with Image J software (National Institutes of Health, Bethesda, MD). The results are adjusted to actin controls, on the same blot, set at 100%, and by gp91^{phox}/actin, caspase-3/actin densitometric ratio. The quantification of the actin guarantees equal protein loading of the immunoblot.

Cellular glutathione assay. Total glutathione [GSSG (oxidized form of glutathione) and GSH (reduced form of glutathione)] levels were measured in H9c2 cells using a glutathione assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's directions. Briefly, glutathione and 5,5′-dithio-bis-2-nitrobenzoic acid react to generate 5-thio-2-nitrobenzoic acid. The rate of 5-thio-2-nitrobenzoic acid production is directly proportional to the concentration of glutathione in the sample. The concentration of glutathione in the sample solution was determined by measuring absorbance at 405 nm. For quantification of GSSG, cell lysates were treated with 2-vinylpyridine and triethanolamine to block the sulfhydryl residue of GSH. The levels of GSSG were determined photometrically as described previously for GSH. Total glutathione and GSSG levels were expressed as nanomoles per milligram protein. The redox ratio was calculated as the ratio of GSH, determined by subtracting GSSG from total glutathione levels, to GSSG.

Annexin V-phycoerythrin (PE) and 7-AAD staining apoptosis assay. PE-conjugated annexin V and 7-AAD (7-amino-actinomycin) labeling were used to detect the externalization of phosphatidylserine (PS) during apoptotic progression in cells exposed to HR (BD Pharmingen, San Diego, CA). After detachment, cells were resuspended in binding buffer (10 mM HEPE/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl$_2$) at a density of 5×10^5 cells/mL and stained. The fluorescence was measured with a FACSCalibur flow cytometer and analyzed using the Cell Quest software (BD Biosciences, San Diego, CA). Seven thousand cells were examined for each sample. The values were expressed as percent of apoptotic cells.

Animal model. This study protocol conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee at the University of Michigan. Neonatal (7–14 d old) rabbit hearts were rapidly excised through a median sternotomy, suspended in a Langendorff apparatus for perfusion with oxygenated Krebs-Henseleit buffer composed of 119 mM

NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 25 mM NaHCO$_3$, and 10 mM glucose, which was gased with 95%O$_2$/5%CO$_2$ (pH 7.4, 38°C). Krebs-Henseleit solution was delivered at a constant rate of 25 to 30 mL/min to establish an initial mean coronary artery perfusion pressure of above 40 mm Hg. A balloon-tipped, fluid-filled catheter attached to a pressure transducer was inserted through the left atrial appendage into the left ventricle (LV) for continuous monitoring of ventricular performance. Initial LV end-diastolic pressure was ~10 mm Hg. Coronary flow rate was maintained at 4 to 8 mL/min to maintain perfusion pressure of 40 to 45 mm Hg, and hearts were allowed to equilibrate for 30 min. Measurements of LV performance, that is, LV developed pressure (LVDP = LV end-systolic pressure − LV end-diastolic pressure) and the rate of pressure development (LV dP/dt), were recorded using a PowerLab data acquisition system (AD Instruments, Australia).

Langendorff-perfused neonatal rabbit hearts were randomly assigned to three study groups: 1) control ($n = 5$): rabbit hearts were continuously perfused with buffer; 2) IR ($n = 3$–5): global ischemic injury was simulated by interruption of coronary perfusion for 15, 30, or 60 min, followed by reperfusion with buffer; and 3) IR + NAC ($n = 4$): rabbit hearts underwent IR protocol with buffer containing 0.4 mM NAC.

Statistical analysis. All data were expressed as mean ± SEM. Differences between two groups were compared by unpaired *t* test. For multigroup comparison, ANOVA followed by the Bonferroni or Fisher's Least Significant Difference (LSD) post hoc test was performed. A value of $p < 0.05$ was considered statistically significant.

RESULTS

NAC attenuates gp91^{phox} and caspase-3 protein expression in HR-induced H9c2 cells. To investigate whether ROS production plays a role in HR-induced apoptosis, H9c2 cells were exposed to varying lengths of hypoxia (0, 6, 12, 18, 24 h) followed by reoxygenation and gp91^{phox} and caspase-3 protein levels were analyzed. Levels of gp91^{phox} started to increase at 6 h, reached maximum by 12 h (0.21 ± 0.05 relative units, $p < 0.05$ *versus* control) and were sustained for up to 24 h after HR treatment (Fig. 1*A*). Similarly, caspase-3 expression was increased at 6 h and maximum at 12-h HR compared with the control group (2.22 ± 0.07 *versus* 0.72 ± 0.17, relative units, $p < 0.001$; Fig. 1*B*).

To examine whether NADPH oxidase-mediated ROS directly induces apoptosis, H9c2 cells were incubated with NAC (0.25–2.0 mM). After 12 h of hypoxia, gp91^{phox} and caspase-3 levels were measured. Scavenging ROS by NAC inhibited gp91^{phox} and caspase-3 expression in HR-stimulated H9c2 cells. NAC markedly decreased gp91^{phox} expression with a

maximum effect observed at 0.5 mM [from 0.52 ± 0.04 to 0.05 ± 0.02 ($p < 0.001$; Fig. 1C)]. Similarly, caspase-3 expression was significantly attenuated by NAC treatment compared with untreated cardiomyoblasts (1.18 ± 0.24 at 2 mM *versus* 2.97 ± 0.28, $p < 0.001$; Fig. 1D). These results demonstrate that HR activates the caspase-3-dependent apoptotic pathway by NADPH oxidase-mediated ROS-dependent mechanisms in H9c2 cells and confirms that NAC acts as an ROS scavenger in this system.

NAC restored GSH/GSSG ratio reduced by HR. To confirm ROS formation in H9c2 cells exposed to HR, the levels of total GSH and GSSG were measured. GSH serves as a nucleophilic cosubstrate to glutathione transferases in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides (18). A change in cellular GSH level is usually accompanied by a concomitant change in the GSSG levels. Levels of total glutathione significantly decreased in a time-dependent fashion after HR treatment and could not be detected after 24-h HR (Fig. 2A). The GSSG level in the 12-h HR group was significantly increased compared with the control group (7.87 ± 1.03 and 3.69 ± 0.62 nmol/ mg protein, respectively; $p < 0.01$; Fig. 2B). The GSH/GSSG ratio reflects accumulation of GSSG, and thus it more reliably reflects cellular redox status. Consistently, the GSH/GSSG ratio after HR treatment was decreased by 68% at 6 h ($p < 0.001$) and 80% at 12-h HR ($p < 0.001$) compared with the control group (Fig. 2C).

Total glutathione levels after NAC treatment did not differ between groups (Fig. 2D). However, NAC treatment significantly attenuated the GSSG content compared with the HR control group (Fig. 2E). NAC treatment also restored the GSH/GSSG ratio that was reduced after HR treatment. Treatment with 0.5 mM NAC resulted in the maximum GSH/GSSG ratio compared with the HR control group (39.61 ± 6.03 *versus* 4.15 ± 0.51, respectively, $p < 0.001$; Fig. 2F). Interestingly, the GSH/GSSG ratios in the NAC-treated groups were higher than in the normoxic control group, suggesting that NAC maybe provide cells with additional cysteine for GSH synthesis. These results demonstrate that HR induces oxidative stress through glutathione-dependent redox regulation in H9c2 cells and confirms that NAC is able to restore the GSH/GSSG ratio that was reduced by HR.

NAC treatment attenuated the H9c2 cells apoptosis induced by HR. To further characterize and quantify the degree and mode of cell death during HR and the potentially protective effects of NAC, apoptotic, necrotic, and living cells were differentiated and quantified by flow cytofluorometry.

Flow cytometric analysis of annexin V/7-AAD staining of H9c2 cells exposed to 12-h HR showed that $22.9 \pm 1.9\%$ ($p < 0.001$ *versus* control) of the cells were in the early apoptotic stage, and only 5% were necrotic (Fig. 3A and B). Exposure of cells to HR for 18 h produced more necrosis ($27.5 \pm 2.0\%$, $p < 0.001$ *versus* control; Fig. 3B). Treatment of H9c2 cells with NAC (0.25–2 mM) for 12 h reduced the percentage of apoptotic cells in a concentration-dependent manner from $21.6 \pm 1.3\%$ to $13.2 \pm 3.1\%$ (0.5 mM), $11.4 \pm 1.2\%$ (1 mM), and $9.6 \pm 1.0\%$ (2 mM) (Fig. 3C). NAC treatment did not significantly affect necrosis compared with untreated H9c2 cells (Fig. 3D). These results indicate that both apoptosis and necrosis contribute to cell death after HR, and NAC treatment only attenuates apoptosis.

IR injury reduced LV function and increased the expression of gp91^{phox} and caspase-3 in neonatal rabbit hearts. To evaluate the relationship between IR-induced ROS production, apoptosis, and ventricular dysfunction, we investigated the effects of IR in an isolated neonatal rabbit heart model. A brief period of ischemia (15 min) followed by reperfusion had no significant adverse effect on left ventricular function. How-
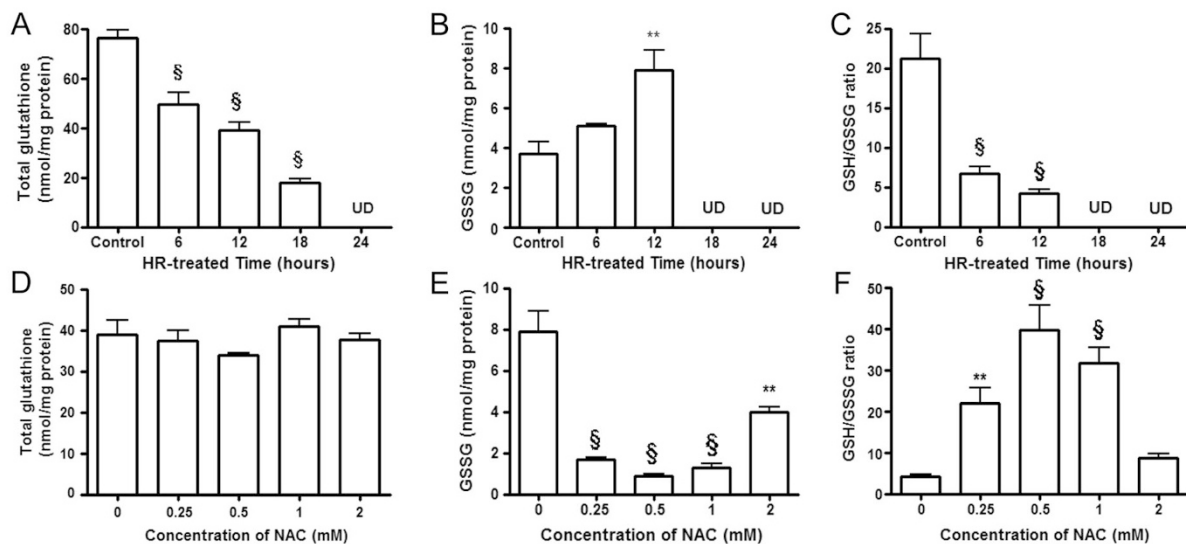

Figure 2. Total glutathione, GSSG levels, and GSH/GSSG ratio in H9c2 cells in response to HR and NAC treated. H9c2 cells were exposed to HR for 6, 12, 18, and 24 h. Total glutathione and GSSG (nmole/mg protein) levels were measured (*A* and *B*). The GSH/GSSG ratio was calculated by subtracting GSSG from total glutathione levels, to GSSG (*C*). Then H9c2 cells were treated with different concentration of NAC (0.25–2.0 mM) or vehicle for 12 h HR. Total glutathione and GSSG levels were measured (*D* and *E*). The GSH/GSSG ratio was calculated (*F*). Data were mean $\pm$ SE from four or five independent experiments performed in duplicate. **$p < 0.01$, §$p < 0.001$ *vs* control. UD indicates undetectable levels.
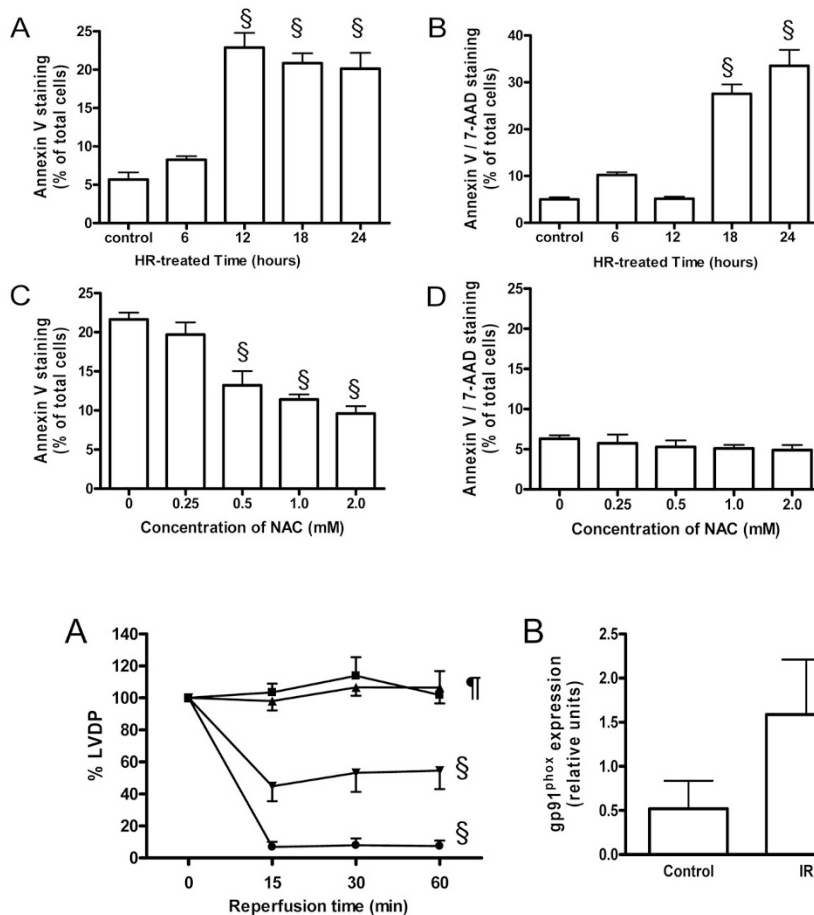
Figure 3. Measurement of apoptosis and necrosis in HR-induced and NAC-treated H9c2 cells. H9c2 cells were induced by HR for 6, 12, 18, and 24 h. Apoptosis and necrosis were assessed by flow cytometric analysis of PE-annexin V and 7-AAD nuclear staining. The apoptotic cells stained with annexin V (*A*) and necrotic cells stained with annexin V/7-AAD (*B*) are indicated as a percentage of gated cells. Then, H9c2 cells treated for 12 h HR with different concentration of NAC (0.25–2 mM) or vehicle control were processed for staining with annexin V-positive cells (*C*) and annexin V/7-AAD duel positive cells (*D*). Data are mean ± SE of three or four different experiments. §*p* < 0.001 *vs* control.
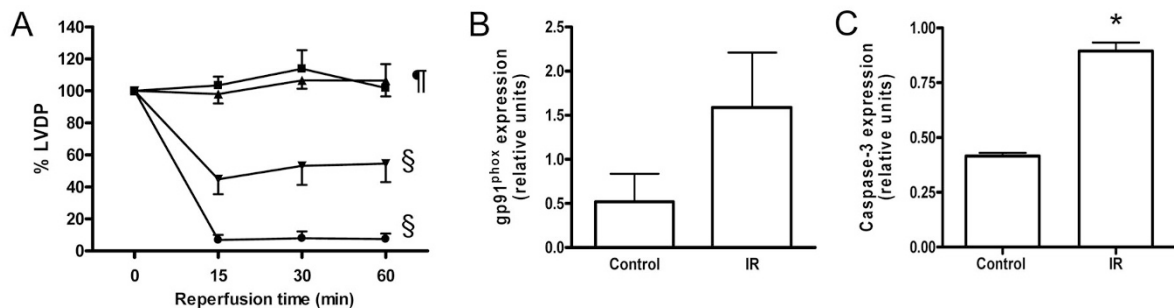

Figure 4. Cardiac function during reperfusion after IR and protein expression of gp91^{phox} and caspase-3 in neonatal rabbit hearts. (*A*) Langendorff-perfused hearts subjected to 15 (*n* = 3, ▲), 30 (*n* = 5, ▼), and 60 min (*n* = 3, ○) of ischemia followed by 15, 30, and 60 min of reperfusion were measured by LVDP. Control group had no ischemia (*n* = 5, ■). Values were normalized to control. Levels of gp91^{phox} (*B*) and caspase-3 (*C*) in rabbit heart homogenates were measured by immunoblot analysis. Both gp91^{phox} (*n* = 5) and caspase-3 (*n* = 3) protein levels were increased in 30 min of IR compared with control (*n* = 4). Data are expressed as mean ± SE. ¶*p* = 0.842, §*p* < 0.001, **p* = 0.003 *vs* control.

ever, LVDP was significantly reduced by both 30 min (55.4 ± 12.0%, *p* < 0.001) and 60 min (7.4 ± 3.0%, *p* < 0.001) of ischemia followed by reperfusion compared with the control group (Fig. 4*A*).

To investigate a potential mechanism for LV dysfunction after IR injury, lysates of Langendorff-perfused rabbit hearts were analyzed by the Western blot technique for detection of gp91^{phox} and caspase-3. These data showed that the expression of gp91^{phox} and caspase-3 were increased after 30 min of IR compared with control (gp91^{phox}: 1.59 ± 0.62 *versus* 0.52 ± 0.32 relative units, caspase-3: 0.89 ± 0.04 *versus* 0.41 ± 0.004 relative units, *p* = 0.003; Fig. 4*B* and *C*). These results suggest that IR injury significantly attenuates the recovery of LVDP in neonatal rabbit hearts *via* a caspase-3/NADPH oxidase-mediated pathway.

Reperfusion with NAC attenuated myocardial dysfunction after IR. To evaluate the effect of NAC on the postischemic cardiac function, hearts subjected to global ischemia were reperfused with Krebs-Henseleit buffer in the presence or absence of NAC (0.4 mM). NAC treatment significantly improved the recovery of LVDP after 30 min of ischemia compared with untreated hearts (102.0 ± 32.0% *versus*

55.4 ± 12.0%, respectively, *p* = 0.043). NAC markedly attenuated IR-induced myocardial dysfunction and was cardioprotective (Fig. 5). This result indicates that treatment with NAC effectively produces cardioprotection against IR injury.

DISCUSSION

Repair of congenital heart defects in infancy is becoming increasingly common at major pediatric heart centers. Postoperative low cardiac output due to ventricular failure is a major cause of morbidity and mortality often despite a technically successful surgical procedure (19). Recent clinical and experimental studies suggest that ventricular failure is partly due to global IR injury associated with cardiopulmonary bypass and cardioplegic arrest. Myocardial IR injury leads to oxygen-derived free radical production, membrane lipid peroxidation, and impaired postbypass contractility (20,21). Elevated oxygen-derived free radical production initiates and/or promotes apoptotic cascades leading to cell death and tissue damage (22). In this study, we showed that NAC, a potent antioxidant, attenuates NADPH oxidase-mediated ROS production and caspase-3 expression, decreases the percentage of
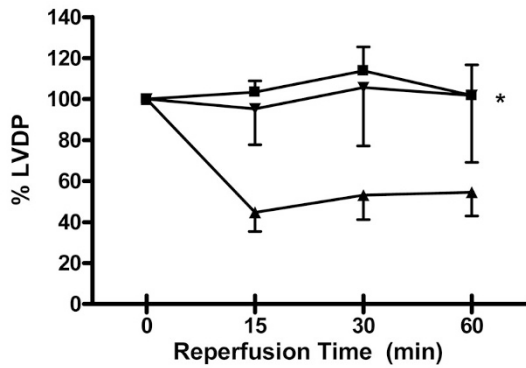
Figure 5. Effect of NAC on IR-induced cardiac dysfunction in neonatal rabbit hearts. Isolated rabbit hearts subjected to 30 min ischemia followed by 15, 30, and 60 min of reperfusion in the presence or absence of NAC (0.4 mM). NAC treatment significantly improved the recovery of LVDP ($n = 4$, ▼) compared with untreated hearts ($n = 5$, ▲). Control group had no ischemia ($n = 5$, ■). Data are normalized to control and expressed as mean ± SE *$p = 0.043$ vs untreated group.

annexin V-positive cells, and restores the glutathione redox ratio in cardiomyocytes exposed to HR. We also demonstrated that NAC prevents apoptosis and myocardial dysfunction in experimental neonatal IR injury.

NADPH oxidase is a membrane-associated, multisubunit enzyme complex that has been shown to be a major source of ROS in myocardium. Caspase-3 plays a pivotal role in the execution of apoptosis. Furthermore, glutathione is the most abundant cellular thiol and plays a central role in maintaining cellular redox balance. Under both HR and IR conditions, NAC seems to inhibit NADPH oxidase activation and ROS production and restores the glutathione redox ratio leading to prevention of caspase-3-dependent apoptosis and myocardial dysfunction. Our results support the protective role of NAC and are fully consistent with the hypothesis that NAC can reduce IR injury associated with ROS-mediated apoptosis and improve cardiac functional recovery in neonatal cardiomyocytes. These results are consistent with the protective effects of NAC in primary rodent cardiac myocytes (23) and may have important potential therapeutic implications for young children undergoing heart surgery with cardiopulmonary bypass and cardioplegic arrest.

Cardiomyocyte apoptosis is one of the major pathogenic mechanisms underlying myocardial IR injury. This study showed that myocardial IR injury in newborn rabbits led to myocardial dysfunction and increased NADPH oxidase and caspase-3 expression. These results are consistent with our *in vitro* data using cultured cardiomyocytes exposed to HR. We confirm that ROS generated by HR or IR are important in initiating the series of pathological events causing cell death through apoptosis. In addition, ROS may cause direct damage to cardiomyocytes and contribute to the pathogenesis of myocardial injury during IR.

The H9c2 cell line derived from embryonic rat hearts maintains several features of cardiac myocytes and has been used as an *in vitro* model to study the effects of various medications on cardiac cells (24,25). Therefore, we also used H9c2 cells as an *in vitro* model. In this study, cultured

cardiomyocytes subjected to HR showed a time-dependent increase in NADPH oxidase, caspase-3 protein expression, and GSSG levels. HR significantly reduced the glutathione redox ratio, and flow cytometry confirmed that the early phase of HR-mediated cell death was primarily due to apoptosis, not necrosis. We are currently performing experiments that will allow us to extend these observations to freshly dispersed cardiomyocytes in culture.

NAC has several uses in both pediatric and adult medicine. NAC has been used as an inhalational mucolytic agent in bronchopulmonary illnesses including cystic fibrosis, as a renoprotective agent for contrast-induced nephropathy, and as an antidote for liver injury from acute acetaminophen toxicity (26). These are important clinical applications that have helped established that NAC administration is safe in humans and suggest that NAC merits further investigation as a therapeutic strategy for adults and children at risk for IR injury. Recently, results from a pilot study of 20 neonates with d-transposition of the great arteries showed that perioperative treatment with NAC attenuates postoperative renal dysfunction and intensive care unit length of stay after arterial switch operation (5). On the basis of this clinical trial and our current experimental results, a large, multiinstitutional randomized controlled trial of NAC in patients with congenital heart disease undergoing cardiac surgery seems warranted.

Neonatal hearts respond to oxidative stresses quite differently than adult hearts (27). Saugstad and coworkers reported that newborns, particularly premature infants, are more susceptible to oxidative stress than older children and adults because of immature native antioxidant systems. Conversely, other investigators suggested that fetal and neonatal hearts have adaptive mechanisms that make them less vulnerable to hypoxia and ischemia compared with the adult heart (28–30). Although there are conflicting data regarding the relative susceptibility of immature hearts to IR injury relative to mature hearts, our data demonstrate the key role of ROS in myocardial apoptosis and dysfunction in neonates. A similar role for ROS in IR injury has been reported in adults (31).

Although our *in vitro* and *ex vivo* data indicate that ROS-mediated apoptosis and myocardial dysfunction occur during HR/IR in embryonic rat myocyte and neonatal rabbit studies, this study has some limitations. First, H9c2 cardiomyoblasts are different from primary heart cells. Although this continuous, dedifferentiated cell line displays many morphological characteristics similar to immature embryonic cardiomyocytes, there are clear differences that might affect susceptibility and the various signaling pathways responsible for HR-induced cell injury. In addition, different cell culture conditions can dramatically alter cell metabolism that may affect the response to stress (32). Second, the *ex vivo* isolated Langendorff heart preparation differs significantly from an *in vivo* model because of the lack of an intact circulatory system and an absence of neurohormonal influences. For example, one recent study suggested that IR injury induces a broad change of plasma metabolites that may affect ventricular function (4).

In summary, this study demonstrates a pivotal role for ROS in mediating cardiomyocyte apoptosis during IR injury.

NADPH oxidase generates ROS that lead to caspase-3-dependent apoptotic cell death and myocardial dysfunction. NAC exerts cardioprotection against myocardial IR injury by NADPH oxidase-mediated and glutathione-dependent redox regulation of apoptosis. This antiapoptotic effect of NAC may help provide biological end-points for the clinical evaluation of NAC as a cardiovascular therapeutic in both infants and adults.

REFERENCES

1. Baker JE 2004 Oxidative stress and adaptation of the infant heart to hypoxia and ischemia. Antioxid Redox Signal 6:423–429
2. Kumar D, Lou H, Singal PK 2002 Oxidative stress and apoptosis in heart dysfunction. Herz 27:662–668
3. Saugstad OD 2005 Oxidative stress in the newborn—a 30-year perspective. Biol Neonate 88:228–236
4. Solberg R, Enot D, Deigner H-P, Koal T, Scholl-Burgi S, Saugstad OD, Keller M 2010 Metabolomic analyses of plasma reveals new insights into asphyxia and resuscitation in pigs. PLoS ONE 5:e9606
5. Aiyagari R, Gelehrter S, Bove EL, Ohye RG, Devaney EJ, Hirsch JC, Gurney JG, Charpie JR 2010 Effects of *N*-acetylcysteine on renal dysfunction in neonates undergoing the arterial switch operation. J Thorac Cardiovasc Surg 139:956–961
6. Whitelaw A, Thoresen M 2002 Clinical trials of treatments after perinatal asphyxia. Curr Opin Pediatr 14:664–668
7. Oliveira MS, Floriano EM, Mazin SC, Martinez EZ, Vicente WV, Peres LC, Rossi MA, Ramos SG 2011 Ischemic myocardial injuries after cardiac malformation repair in infants may be associated with oxidative stress mechanisms. Cardiovasc Pathol 20:e43–e52
8. Xiao L, Pimentel DR, Wang J, Singh K, Colucci WS, Sawyer DB 2002 Role of reactive oxygen species and NAD(P)H oxidase in α1-adrenoceptor signaling in adult rat cardiac myocytes. Am J Physiol Cell Physiol 282:C926–C934
9. Li Y, Arnold MO, Pampillo M, Babwah AV, Peng T 2009 Taurine prevents cardiomyocyte death by inhibiting NADPH oxidase-mediated calpain activation. Free Radic Biol Med 46:51–61
10. Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ 2002 Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. Diabetes 51:1938–1948
11. Song JQ, Teng X, Cai Y, Tang C-S, Qi Y-F 2009 Activation of Akt/GSK-3β signaling pathway is involved in intermedin1–53 protection against myocardial apoptosis induced by ischemia/reperfusion. Apoptosis 14:1061–1069
12. Zafarullah M, Li WQ, Sylvester J, Ahmad M 2003 Molecular mechanisms of *N*-acetylcysteine actions. Cell Mol Life Sci 60:6–20
13. El-Hamamsy I, Stevens LM, Carrier M, Pellerin M, Bouchard D, Demers P, Cartier R, Page P, Perrault LP 2007 Effect of intravenous *N*-acetylcysteine on outcomes after coronary artery bypass surgery: a randomized, double-blind, placebo-controlled clinical trial. J Thorac Cardiovasc Surg 133:7–12
14. Tolar J, Orchard PJ, Bjoraker KJ, Ziegler RS, Shapiro EG, Charnas L 2007 *N*-acetyl-L-cysteine improves outcome of advanced cerebral adrenoleukodystrophy. Bone Marrow Transplant 39:211–215
15. Abe M, Takiguchi Y, Ichimaru S, Tsuchiya K, Wada K 2008 Comparison of the protective effect of *N*-acetylcysteine by different treatments on rat myocardial ischemia-reperfusion injury. J Pharmacol Sci 106:571–577
16. Khanna G, Diwan V, Singh M, Singh N, Jaggi AS 2008 Reduction of ischemic, pharmacological and remote preconditioning effects by an antioxidant *N*-acetyl cysteine pretreatment in isolated rat heart. Yakugaku Zasshi 128:469–477
17. Johnson ST, Bigam DL, Emara M, Obaid L, Slack G, Korbutt G, Jewell LD, Aerde JV, Cheung P-Y 2007 *N*-acetylcysteine improves the hemodynamics and oxidative stress in hypoxic newborn pigs reoxygenated with 100% oxygen. Shock 28:484–490
18. Urata Y, Ihara Y, Murata H, Goto S, Koji T, Yodoi J, Inoue S, Kondo T 2006 17β-estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardiac H9c2 cells. J Biol Chem 281:13092–13102
19. Ma M, Gauvreau K, Allan CK, Mayer JE, Jenkins KJ 2007 Causes of death after congenital heart surgery. Ann Thorac Surg 83:1438–1445
20. Caputo M, Mokhtari A, Rogers CA, Panayiotou N, Chen Q, Ghorbel MT, Angelini GD, Parry AJ 2009 The effects of normoxic versus hyperoxic cardiopulmonary bypass on oxidative stress and inflammatory response in cyanotic pediatric patients undergoing open cardiac surgery: a randomized controlled trial. J Thorac Cardiovasc Surg 138:206–214
21. Ihnken K, Morita K, Buckberg G 1998 Delayed cardioplegic reoxygenation reduces reoxygenation injury in cyanotic immature hearts. Ann Thorac Surg 66:177–182
22. Ramlawi B, Feng J, Mieno S, Szabo C, Zsengeller Z, Clements R, Sodha N, Boodhwani M, Bianchi C, Sellke FW 2006 Indices of apoptosis activation after blood cardioplegia and cardiopulmonary bypass. Circulation 114:I257–I263
23. Fu Z, Guo J, Jing L, Li R, Zhang T, Peng S 2010 Enhanced toxicity and ROS generation by doxorubicine in primary cultures of cardiomyocytes from neonatal metallothionein-I/II null mice. Toxicol In Vitro 24:1584–1591
24. Kumar S, Sitaswad SL 2009 *N*-acetylcysteine prevents glucose/glucose oxidase-induced oxidative stress, mitochondrial damage and apoptosis in H9c2 cells. Life Sci 84:328–336
25. Shi R, Huang CC, Aronstam RS, Ercal N, Martin A, Huang YW 2009 *N*-acetylcysteine amide decreases oxidative stress but not cell death induced by doxorubicin in H9c2 cardiomyocytes. BMC Pharmacol 9:7
26. Walson PD, Groth JF 1993 Acetaminophen hepatotoxicity after a prolonged ingestion. Pediatrics 91:1021–1022
27. Scholz TD, Segar JL 2008 Cardiac metabolism in the fetus and newborn. NeoReviews 9:e109–e118
28. Ihnken K 2000 Myocardial protection in hypoxic immature hearts. Thorac Cardiovasc Surg 48:46–54
29. Mühlfeld C, Urru M, Rümelin R, Mirzaie M, Schöndube F, Richter J, Dörge H 2006 Myocardial ischemia tolerance in the newborn rat involving opioid receptors and mitochondrial K+ channels. Anat Rec A Discov Mol Cell Evol Biol 288:297–303
30. Milerova M, Charvatova Z, Skarka L, Ostadalova I, Drahota Z, Fialova M, Ostadal B 2010 Neonatal cardiac mitochondria and ischemia/reperfusion injury. Mol Cell Biochem 335:147–153
31. Redout EM, Wagner MJ, Zuidwijk MJ, Boer C, Musters RJ, Hardeveld C, Paulus WJ, Simonides WS 2007 Right-ventricular failure is associated with increased mitochondrial complex II activity and production of reactive oxygen species. Cardiovasc Res 75:770–781
32. Banerjee I, Fuseler JW, Price RL, Borg TK, Baudino TA 2007 Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. Am J Physiol Heart Circ Physiol 293:H1883–H1891