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Cardioplegia and Angiotensin II Receptor Antagonists Modulate Signal Transducers and Activators of Transcription Activation in Neonatal Rat Myocytes

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Abstract: Previous investigations have shown that the signal transducers and activators of transcription (STATs) signaling pathway play an important role in the modulation of apoptosis after ischemia and reperfusion. The mechanism for this enhanced cardioprotection is unknown, but we believe that alterations STATs may play a role. To investigate this hypothesis, we examined the effects of angiotension II type 1 (AT1) and angiotension II type 2 (AT2) receptor antagonist added to cardioplegia on the downstream response of different STATs, connected with proinflammatory pathways (STAT2, STAT5) and prohypertrophic and antiapoptotic pathways (STAT3). Isolated, nonworking hearts ($n = 3$ per group) from neonatal rats were perfused aerobically (4°C) for 20 min in the Langendorff mode with the modified St. Thomas' Hospital no. 2 (MSTH2) cardioplegic solution (Group 1), the MSTH2 cardioplegic solution + AT1 receptor antagonist (Group 2), and MSTH2 cardioplegic solution + AT2 receptor antagonist (Group 3). Thus, myocytes were isolated by enzymatic digestion, and STAT2, STAT3, and STAT5 were investigated in Western blot studies. Times to arrest after cardioplegia were 8–12 s for all groups. Total cardioplegia delivery volume was about 300 mL for the 20 min. Perfusion with the MSTH2 cardioplegic solution supplemented with AT1 receptor antagonist (Group 2) induced a significant reduction in STAT2 and STAT5 tyrosine phosphorylation (–58 and –63%, respectively, vs. Group 1, $P < 0.05$). Conversely, STAT2 and STAT5 activation were unaffected by perfusion with the MSTH2 cardioplegic solution supplemented with AT2 receptor antagonist (Group 3). The decreased activation of STAT2 and STAT5 observed in Group 2 was accompanied by reduction of interleukin-1β (–57% in Group 2 vs. Group 1, $P < 0.05$). There were no significant differences in STAT3 phosphorylation among all groups. Only the addition of AT1 receptor antagonist to MSTH2 cardioplegia significantly decreases the inflammatory response of the neonatal rat cardiomyocytes without affecting antiapoptotic influence provided by tyrosine phosphorylation of STAT3. AT1 receptor antagonist added to cardioplegia represents an additional modality for enhancing myocardial protection during cardiac surgery and could contribute to optimize the ischemia tolerance of the pediatric heart. **Key Words:** Rat myocardium—Cardioplegia—Angiotensin II antagonist—Valsartan—Signal transducers and activators of transcription.

Although the tolerance of immature myocardium to ischemia is greater than that of mature myocardium, inadequate protection by crystalloid cardioplegia(s) for immature myocardium has been cited as one of the major reasons for postoperative complications and deaths in children (1).

The interpretation of studies of cardioplegic efficacy in the immature myocardium is complicated by the variety of animal species, the range of different cardioplegic solutions used, and different hypothermias. Therefore, a considerable controversy surrounds the ability of cardioplegic solutions to protect the immature myocardium (2).

Absolutely high potassium (K^+) cardioplegia has been identified as a major factor associated with poor outcomes in pediatric congenital, adult revascularization, or valvular corrective operations (3).

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K^+-induced depolarization and the resulting intracellular sodium (Na^+) and calcium (Ca^{2+}) loading can lead to myocardial and microvascular injury, coronary vasoconstriction and spasm (4), arrhythmias (5), and right and left ventricular stunning (6), and potentiate the local inflammatory response with free radical production (7) (excepting from electrochemical arrest, hyperkalemic cardioplegia offers the patient little or no cardioprotection).

Moreover, the inflammatory reaction to cardiopulmonary bypass and surgical trauma involves activation of leukocytes and endothelium, with secretion of cytokines that contribute to the systemic inflammatory response syndrome, multiple organ failure, and postoperative myocardial motion abnormalities that are frequently observed after cardiac surgery (8).

To minimize cell injury through oxidative stress, it is necessary to either reduce production of oxygen radicals, scavenge oxygen radicals effectively, or block the conversion of reactive species of low reactivity (e.g., hydrogen peroxide) to species of high reactivity (e.g., hydroxyl radicals).

A direct link was found among angiotensin II, reactive oxygen species (ROS) generation, glucose, and the transmission of death signaling in rat myocytes (p38 mitogen-activated protein kinase) (9). Previous studies have shown that the signal transducers and activators of transcription (STATs) signaling pathways activated by angiotensin II play an important role in the modulation of apoptosis after ischemia and reperfusion.

To investigate the hypothesis according to selective angiotensin II antagonism can regulate STATs activation during cardioplegia, we examined the effects of STATs modulation after infusion of modified St. Thomas' Hospital no. 2 (MSTH2) cardioplegia with addition of angiotensin II type 1 (AT1) and type 2 (AT2) receptor antagonist, in particular related with prohypertrophic and antiapoptotic pathways (STAT3) and with proinflammatory pathways (STAT2, STAT5).

MATERIALS AND METHODS

Animals

Neonatal Wistar rats (1–2 weeks) were fed ad libitum and housed in a 14/10-h light/dark cycle. Rats were anesthetized with an intraperitoneal injection of thiopentone sodium (100 mg/kg body weight), and hearts were rapidly excised. Rats were handled in compliance with Verona University Ethics approval and with the "Guide for care and use of laboratory animals" from the National Institutes of Health. Chemicals were obtained from Sigma-Aldrich Italia (Milan, Italy).

Langendorff perfusion system

The chest was opened by a median sternotomy, and the heart was rapidly excised and placed in ice-cold perfusion medium. The time taken from opening the chest to excision of the heart was 1–2 min. Immediately after, the aorta was connected to a standard Langendorff apparatus and perfused with arresting solutions in anterograde fashion at 80 cm H_2O (60 mm Hg). After infusion, hearts were switched rapidly to nonworking mode, and cardioplegia was managed for a total of 20 min at controlled temperature of 4°C (Checktemp Thermometer, LSS, Janesville, WI, USA). Arresting solutions were continuously oxygenated before and during administration.

Arresting solutions

MSTH2 cardioplegia

MSTH2 is a sterile, nonpyrogenic, essentially isotonic, formulation of electrolytes in water for injection. It is a core solution intended for use only after addition of sodium bicarbonate to adjust pH prior to administration. After buffering with sodium bicarbonate, it is suitable for cardiac instillation (usually with hypothermia) to induce arrest during open heart surgery.

Each 100 mL of solution contains calcium chloride dihydrate 17.6 mg, magnesium chloride hexahydrate 325.3 mg, KCl 119.3 mg, and NaCl 643 mg in water for injection. It may contain HCl or NaOH for pH adjustment. Electrolyte content per liter (not including ions for pH adjustment): Ca^{++} 2.4 mEq; magnesium (Mg^{++}) 32 mEq; K^+ 16 mEq; Na^+ 110 mEq; chloride (Cl^-) 160 mEq. Osmolar concentration, 304 mOsmol/L (calc.); pH 3.8 (3.5 to 3.9) prior to sodium bicarbonate ($NaHCO_3$) addition.

It is required that 10 mL (840 mg) of 8.4% $NaHCO_3$ injection (10 mEq each of sodium and bicarbonate) be added aseptically and thoroughly mixed with each 1000 mL of cardioplegic solution to adjust pH. Ten mL 8.4% of $NaHCO_3$ injection should be used to achieve the approximate pH of 7.8 when measured at room temperature. Use of any other $NaHCO_3$ injection may not achieve this pH due to the varying pHs of $NaHCO_3$ injections. Due to its inherent instability with other components, $NaHCO_3$ must be supplemented just prior to administration. After this addition, the solution must be stored under refrigeration and be used within 24 h.

The buffered admixture contains the following electrolytes (per liter): Ca^{++} 2.4 mEq, Mg^{++} 32 mEq, K^{+} 16 mEq, Na^{+} 120 mEq, Cl^{-} 160 mEq, and HCO^{3-} 10 mEq; osmolar concentration, 324 mOsmol/L (calc.); pH 7.8 (approx.). If other agents are added, these values may be altered.

MSTH2 cardioplegia plus AT1 and AT2 receptor antagonists

To investigate the STATs modulation as consequence of the relative role of the two AT receptor subtypes, selective AT1 (Valsartan, 1 μmol/L) and AT2 (PD123319, 1 μmol/L) receptor antagonists were added to MSTH2 cardioplegia prior to instillation. Ultimate solutions were indicated as MSTH2 cardioplegia + AT1 and MSTH2 cardioplegia + AT2, respectively.

Infusion protocol

Neonatal rats were randomly assigned to three different groups. Group 1 was perfused with MSTH2 for 20 min at 4°C, in anterograde fashion at 80 cm H_2O (60 mm Hg) in the Langendorff perfusion system. The time range of 20 min was chosen to exceed the usual maximum storage time of human hearts, thus creating stiff testing conditions. Group 2 and Group 3 were perfused in the same way with MSTH2 cardioplegia + AT1 and MSTH2 cardioplegia + AT2 solutions, respectively.

Rat heart isolation

Myocytes were isolated with the enzymatic digestion method as previously described (10–13).

Briefly, the aorta previously cannulated for cardioplegia administration was perfused for 10 min at 37° with a calcium-free basic buffer (blood washout) composed of Joklik modified minimal essential medium (MEM Joklik) supplemented with glutamine (0.3 g/L), taurine (1.25 g/L), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (2.9 mmol/L), insulin (20 U/L), penicillin-streptomycin (50 U/mL and 0.05 mg/mL, respectively) 5 mL/L, and $CaCl_2$ (7.5 mmol/L), pH 7.4. The basic buffer was previously passed through filters of 0.2-mm pore size. Perfusion was then switched to collagenase solution, composed of 0.5 mg/mL of collagenase type II (100 units/mL) in basic buffer supplemented with $CaCl_2$ 30 mmol/L (20 mL/min for 20–25 min). The collagenase-perfused tissue was then minced, and tissue was collected in tubes containing basic buffer supplemented with bovine serum albumin (0.5%), $CaCl_2$ (0.3 mmol/L), and taurine (10 mmol/L). Individual myocytes were then released from the tissue by mechanical agitation.

The suspension was filtered through sterile gauze to separate cells from tissue mass. The populations of cells were then washed using two complete cycles of low-speed centrifugation. The dispersed cells were finally preplated for 30 min to minimize fibroblast contamination. Typical preparations contained 60–70% rod-shaped, quiescent Ca^{2+}-tolerant myocytes that had well-defined, regular cross-striations and sarcomere patterns. According to their appearance under phase contrast microscopy and by immunocytochemical staining, nonmyocyte cells were found to account for less than 2% of the total cells (13–15).

Samples were washed in ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 1 mmol/L sodium orthovanadate, 100 mmol/L NaF, 2 mmol/L ethylene glycol tetraacetic acid. 1% NP40 (Tergitol Solution, Sigma-Aldrich Italia), 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4) supplemented with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail. Lysates were kept on ice, sonicated four times for 5 s, and centrifuged at 13 000 × *g* for 10 min. After centrifugation, protein concentration in the supernatant fraction was assessed by Bradford's method.

Western blotting studies

Samples (25 mg proteins) were then separated with 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gel was then transferred to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Piscataway, NJ, USA) and blocked by incubation for 1 h at room temperature in Tris Tween buffered saline (TTBS, pH 7.4) and 5% skimmed milk powder. Membranes were then incubated overnight at 4°C with polyclonal rabbit anti-mouse STAT2 (Santa Cruz Biotechnology), polyclonal rabbit anti-mouse phospho-STAT2 (Tyr690) (Cell Signaling Technology, Danvers, MA, USA), polyclonal rabbit anti-mouse STAT3, polyclonal rabbit anti-mouse phospho-STAT3 (Tyr705) (Cell Signaling Technology), polyclonal rabbit anti-mouse STAT5 and polyclonal rabbit anti-mouse phospho-STAT5 (Tyr694) (Cell Signaling Technology), and polyclonal rabbit anti-mouse interleukin (IL)-1β (Cell Signaling Technology). The nitrocellulose membranes were then washed twice for 10 min with TTBS and incubated for 30 min with goat anti-rabbit or sheep anti-mouse secondary antibodies labeled with horseradish peroxidase. After extensive washing, the specific bound antibody was visualized using a chemiluminescent detection system (ECL detection reagents, Amersham Bioscience). The amount of each band

was quantified using a densitometer software (Quantity One, Bio-Rad, Hercules, CA, USA) and normalized using the total protein amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Data are expressed as mean ± standard deviation. Comparisons between groups were performed using one-way analysis of variance and Student's *t*-test, followed by the Tukey–Kramer method, as appropriate. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Times to arrest after cardioplegia were 8–12 s for all three groups. Total cardioplegia delivery volume was about 300 mL for the 20 min.

Western blot analyses performed on myocytes isolated after infusion according to the protocol's scheme showed that AT1 receptor antagonist significantly reduced STAT2 Tyr690 phosphorylation (–58% in Group 2 vs. Group 1, $P < 0.05$) (Fig. 1) and STAT5 Tyr694 phosphorylation (–63% in Group 2 vs. Group 1, $P < 0.05$) (Fig. 1). Conversely, no change in Tyr705 phosphorylation of STAT3 was observed (Fig. 1). The decreased activation of STAT2 and STAT5 activation in Group 2 was accompanied by considerable reduction of IL-1β (–57% in Group 2 vs. Group 1, $P < 0.05$) (Fig. 2). STAT2 and STAT5 activation was blunted by AT1 receptor antagonism but not AT2, as STAT2 Tyr690 phosphorylation only increased 6% in Group 3 versus Group 1 (P = NS) (Fig. 1) and STAT5 Tyr694 phosphorylation documented decreased 3% in Group 3 versus Group 1 (P = NS).

Therefore, STAT2 and STAT5 responses were all mediated by AT1 receptor subtype, as indicated by the relative effects of Valsartan rather than PD123319 (1 μmol/L for both). Conversely, Tyr705 phosphorylation of STAT3 was unaffected by perfusion with cardioplegia in the presence of AT1 or AT2 receptor antagonists (Fig. 1).

DISCUSSION

The effects of formulation and the physicochemical characteristics of cardioplegic solutions on myocardial protection are substantial. The responsiveness of age-related changes of hearts to ischemia and crystalloid cardioplegias also greatly affect the protection effect.

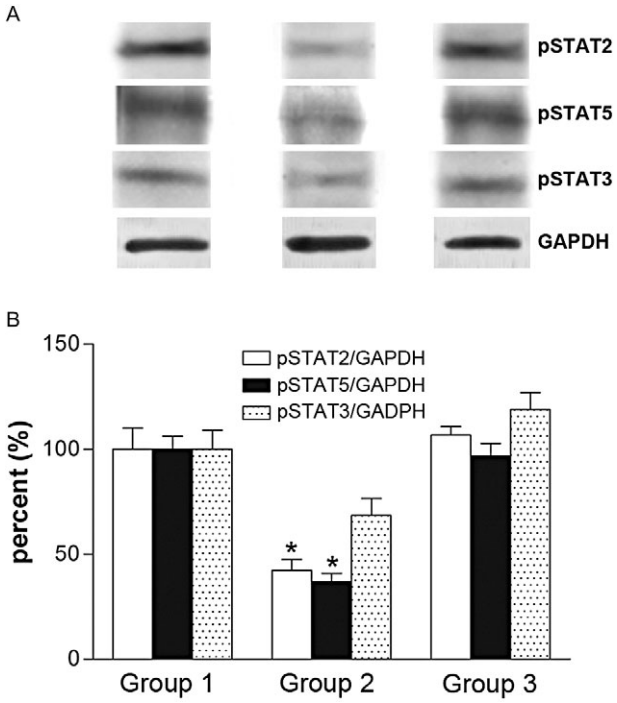

FIG. 1. pSTAT2, pSTAT5, and pSTAT3 expression in myocytes isolated from neonatal rat heart after perfusion with modified St. Thomas Hospital no. 2 (MSTH2) cardioplegia (Group 1), MSTH2 cardioplegia supplemented with AT1 receptor antagonist (Group 2), and with MSTH2 supplemented with AT2 receptor antagonist (Group 3). (A) Representative protein bands of pSTAT2, pSTAT5, pSTAT3, and GAPDH detected by Western blotting. (B) The bar graphs showing the densitometric analysis of the ratio of phosphorylated STAT (pSTAT2, pSTAT5, pSTAT3) to total GAPDH. Data are the results of three experiments for each group of myocytes. *$P < 0.05$ vs. Group 1.

The results of recent studies have demonstrated that crystalloid cardioplegias can indeed confer satisfactory protection on a model of immature rabbit hearts subjected to hypothermic ischemic arrest at 14°C. However, the extent of protection and, hence, the postischemic recovery of function, is highly dependent on the composition of the solution used.

Experimental reports have shown that cardioplegic solutions used at St. Thomas' Hospital and Hopital Lariboisiere provided excellent protection during hypothermic ischemic arrest in neonatal rabbit hearts and resulted in functional recovery superior to that achieved with hypothermia alone or with the high potassium cardioplegic solution (16). Thus, Ledingham et al. have determined the St. Thomas' Hospital solution 2 provides better protection than solution 1; to the best of our knowledge, no clinical evidence has demonstrated superiority of St Thomas' solution 2 over solution 1, and their use is still associated with significant reperfusion injury (14).

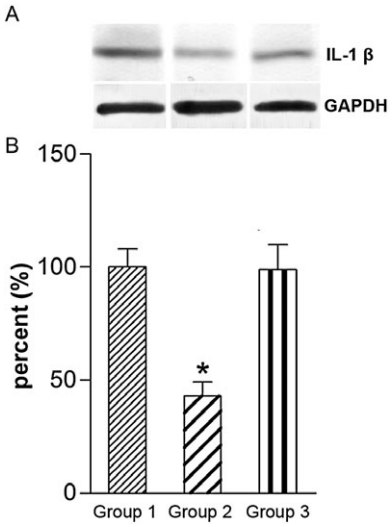

FIG. 2. IL-1β expression in myocytes isolated from neonatal rat heart after perfusion with modified St. Thomas Hospital no. 2 (MSTH2) cardioplegia (Group 1), MSTH2 cardioplegia supplemented with AT1 receptor antagonist (Group 2), and with MSTH2 supplemented with AT2 receptor antagonist (Group 3). (A) Representative protein bands of IL-1β detected by Western blotting. (B) The bar graph showing the densitometric analysis of the ratio of IL-1β to total GAPDH. Data are the results of three experiments for each group of myocytes. $^*P < 0.05$ vs. Group 1.

Although St. Thomas' Hospital or Tyers solutions confer excellent protection on the immature myocardium, other crystalloid solutions as the Bretschneider and Roe solutions are ineffective.

These results always derived from immature rabbit hearts were different from the results of crystalloid cardioplegias on adult hearts. The reasons underlying these differences in cardioplegic efficacy probably include age-related changes in cardiac metabolism, and the way in which components of these solutions interact with the ischemic myocardium. Considerations about the changes among different solutions, and between adult and immature hearts with the same solution, may reveal features of the immature myocardium that could be exploited in the design of protective interventions.

In previous dose–response studies, protection in the adult myocardium was shown to be highly dependent on both the calcium and magnesium content of the St. Thomas' Hospital solution. The comparable protection seen in the present studies with the St. Thomas' Hospital and other crystalloid solutions might, therefore, suggest that the neonatal heart has a different responsiveness to these ions than the adult heart (15,17).

The mechanisms for neonatal cardioprotection are not completely elucidated and partially unknown, but we believe that alterations in STATs signal mediated by changes in electrolytes balance may play a role (18–20).

To further investigate this hypothesis, we have examined the effects of selective AT1 and AT2 receptor antagonist on tyrosine phosphorylation of STAT2, STAT5, and STAT3.

Previous studies have shown that tyrosine phosphorylation of STATs is essential for dimerization and translocation into the nucleus to allow for DNA binding (2). STAT1, STAT2, and STAT5 tyrosine phosphorylation has been shown to be associated with the modulation of apoptosis.

Increased STAT2 tyrosine phosphorylation and STAT1 has been associated with proapoptotic responses in adult rat and human myocardium. Preceding findings have revealed that STAT1-deficient mice develop spontaneous and chemically induced tumors more rapidly than wild-type mice, and that STAT1-deficient cells are more resistant to agents that induce apoptosis (21). It has also been disclosed that STAT1 and STAT2 modulate cardiac myocyte apoptosis after simulated ischemia and reperfusion (22). In contrast, depletion of STAT3 has been shown to abolish the cardioprotection afforded by preconditioning (23).

Our data reveal that STAT2 and STAT5 tyrosine phosphorylation are significantly increased during ischemia in Group 1 as compared with Group 2 (Fig. 1). The mechanism for this can be explained by the downstream of proapoptotic response of STAT2 and STAT5 operated by AT1 receptor antagonist without affecting significantly antiapoptotic STAT3 tyrosine phosphorylation, as evident in Group 1. These data suggest that apoptosis may be the result of a shifting of the balance between proapoptotic and antiapoptotic levels.

We speculate that ischemia induces a growth in proinflammatory STAT2 and STAT5 levels that increases apoptosis. Antiapoptotic STAT3 levels are not significantly decreased among the three groups. This differential leads to a shift in balance toward the reduction of apoptosis by STAT2 and STAT5.

Inhibition of AT2 receptors does not reflect on downstream of proinflammatory STATs, although AT2 receptors have been demonstrated in fetuses (24). We can suppose an AT2 receptor downregulation after birth in the cardiovascular system, but other works need to sustain that hypothesis.

Previous reports have speculated that even the preservation of mitochondrial structure and function by diazoxide added to cardioplegia plays an important role in the modulation of ischemia/reperfusion injury (25). The mechanism through which the

opening of mitochondrial ATP-sensitive K+ channels with diazoxide significantly increases antiapoptotic STAT3 tyrosine phosphorylation levels and DNA binding remains to be fully elucidated. Yoo et al. have shown that c-Jun interacts with STAT3 and enhances STAT3 DNA binding (26).

STATs stimulation reveals on activation of proinflammatory cytokines that play an emerging role as responsible factors for initiating and maintaining myocardial response to environmental stress. The short-term expression of cytokines seems to let the myocardium rapidly react to injury and to activate protective homeostatic responses to damage. However, most recent experimental studies documented the negative immediate inotropic effect on heart function, mainly in case of high levels of tumor necrosis factor, IL-1, IL-2, and IL-6 (27).

In particular, IL-1α and IL-1β are synthesized as 31- to 34-kDa promolecules. They are released from monocytes and macrophages as proteolytically processed 17-kDa mature molecules that bind with high affinity to specific receptors on target cells. IL-1 is not released via the classic secretory pathway. The promolecules are synthesized as cytosolic proteins without signal peptides.

Hogquist et al. demonstrated that release of IL-1 is efficiently induced by cell injury (28). When the injury causes cellular necrosis, IL-1α is released as a mixture of unprocessed and processed molecules, but IL-1β is released exclusively as the biologically inactive proform. In contrast, when cells undergo apoptosis, maturation of both IL-1α and IL-1β is efficient. When apoptosis is rapid, as in macrophages that are targets for allospecific cytotoxic T lymphocytes, processing is observed to occur intracellularly. These findings proposed that cell injury is an important physiologic stimulus for release of IL-1, and nature of the injury profoundly affects the forms of IL-1 that are released.

Furthermore, elevated levels of IL-1 are found after myocardial infarction and are considered to play a major role in tissue remodeling events throughout the heart (29).

The implication of proinflammatory cytokines in cardiac surgery and the relationship to inflammatory reaction due to cardiopulmonary bypass, myocardial arrest, and surgical trauma have been also demonstrated (30).

We first speculated the effects of the AT receptor antagonism in relationship with a specific crystalloid cardioplegia on myocardial stress in the experimental neonatal rat model. The significant decrease of IL-1 level controlled by AT1 receptor antagonism should have the effect of a decrease in myocardial cell ischemic insult due to oxygen-derived free radicals (ROS) local production even in neonatal myocardium.

Myocardial cytokine reduction should be related to protective effects against inflammatory response mediated by proinflammatory STATs activation, with probably less activation of complement leukocytes and endothelial cells, and decreased generation of ROS by polymorphonuclear neutrophils.

Additionally, infusion of moderate hypothermic cardioplegia results in protection against apoptosis.

The role of hypothermia in the prevention of apoptosis has been previously reviewed, and it has been shown that mild hypothermia is somewhat protective, while deep hypothermia increases apoptosis (31). In our model, we administer cold 4°C cardioplegia. No attempt at modulation of temperature beyond these limits has been performed. We have assumed from literature that deep hypothermia may decrease transcription, and thus decrease the antiapoptotic effects of STATs modulation; however, further studies are needed to support this hypothesis.

CONCLUSION

Our data indicate that STAT2 and STAT5 tyrosine phosphorylation are associated with increased apoptosis as confirmed by monitoring of IL-1β trend. Only the addition of AT1 receptor antagonist at cardioplegia modulates apoptosis by significantly decreasing tyrosine phosphorylation of STAT2 and STAT5 without significantly affecting STAT3 response. It represents an additional modality for enhancing neonatal myocardial protection above all for patients at greater risk to suffer from free radical injury when they undergo surgical repair (e.g. tetralogy of Fallot). Our work also raises an interest that the AT2 receptor antagonism does not modulate signaling pathways mediated by STATs in neonatal rat myocardium.

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