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Delayed preconditioning in cardiac myocytes with respect to development of a proinflammatory phenotype: role of SOD and NOS

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Abstract

Objective: Both superoxide dismutase (SOD) and nitric oxide synthase (NOS) have been implicated in delayed preconditioning (DP) to ischemia/reperfusion (I/R) in the heart. We used isolated cardiac myocytes to test the hypothesis that SOD and NOS may interact in the development of DP. **Methods:** Mouse neonatal cardiac myocytes were challenged with anoxia/reoxygenation (A/R; an in vitro counterpart to I/R) and normoxia/normoxia (N/N) served as the control. Two indices of inflammation were measured: oxidant stress (DHR oxidation) and polymorphonuclear leukocyte (PMN) transendothelial migration (cell culture inserts). The role of SOD was assessed using an antisense approach and the role of NOS was assessed using iNOS and eNOS deficient myocytes. **Results:** Cardiac myocytes exposed to A/R (1) produced more oxidants (intracellular fluorescence emission from 2.0±0.1 for N/N to 3.0±0.3 for A/R; $P<0.05$) and (2) promoted PMN migration (% migration from 8.4±0.9 for N/N to 14.1±1.1 for A/R; $P<0.05$). DP occurred if the myocytes were pretreated with an A/R challenge 24 h earlier. That is, these A/R-induced responses were significantly reduced (fluorescence emission 1.9±0.1 and % migration 8.4±0.7; $P<0.05$ as compared to A/R with no pretreatment). Myocyte Mn-SOD, but not Cu/Zn-SOD, activity increased 24 h after the initial A/R challenge. A Mn-SOD antisense oligonucleotide prevented the development of DP. DP occurred in iNOS, but not eNOS, deficient myocytes. A/R increased mRNA for eNOS, but not iNOS, in wild-type myocytes. A/R increased Mn-SOD protein in both iNOS and eNOS deficient myocytes. However, Mn-SOD activity increased only in iNOS deficient myocytes. **Conclusions:** Collectively, these findings suggest that Mn-SOD and eNOS may act in concert in the development of DP in cardiac myocytes.

Keywords: Preconditioning; Hypoxia/anoxia; Myocytes; Infection/inflammation; Leukocytes

1. Introduction

A substantial amount of evidence supports the contention that ischemia/reperfusion (I/R)-induced injury to the heart is due, in part, to an acute inflammatory response in the affected tissue [1–4]. A hallmark feature of this inflammatory response is polymorphonuclear neutrophil (PMN) infiltration into the interstitium. Indeed, the severity of myocardial injury induced by I/R is directly related to the extent of PMN accumulation in the tissue [5]. There is some debate, however, over whether PMN infiltration is the cause or result of I/R-induced myocardial injury [1,6,7]. For example, I/R-induced myocardial infarction can be independent of PMN under certain conditions, e.g. prolonged periods of ischemia [6,7]. Furthermore, I/R-induced myocardial infarction can occur in blood-free heart preparations [6]. On the other hand, there is substantial evidence favoring a role for invading PMN in this pathology. Several studies have shown that interfering with PMN infiltration into the myocardial interstitium affords protection against I/R-induced myocardial injury. Experimental maneuvers aimed to this effect include rendering animals neutropenic [1], immunoneutralization (antibodies) of adhesion glycoproteins on PMN or endothelial cells [8–12], and the use of genetically altered mice deficient in PMN or endothelial cell adhesion molecules

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[13,14]. Our approach to circumvent this cause vs. effect issue has been to use experimental models of I/R-induced inflammation which do not directly cause cell injury [15–17].

Paradoxically, challenging the heart with I/R renders the myocardium more resistant to a subsequent I/R insult [18–20]. This phenomenon can be subdivided into two distinct phases based on the time course and mechanisms involved. An early phase of protection becomes evident within a few minutes after the first I/R and persists for only 1–2 h (acute preconditioning). This early phase is independent of protein synthesis and relies on activation of existing effector molecules to manifest its protective effects. There is also a later phase of protection that comes into play 24 h or later after the initial I/R challenge and can persist for up to several days (delayed preconditioning). This later phase of protection represents a genetically mediated adaptational response within the myocardium and is dependent on protein synthesis. The focus of the present study was to dissect out the mechanisms involved in the development of delayed preconditioning, with respect to the induction of a proinflammatory phenotype in cardiac myocytes [16].

Both antioxidant enzymes and nitric oxide synthase (NOS) have been implicated as effector systems in delayed preconditioning [4,18,19,21]. Of the endogenous antioxidant enzymes that are up-regulated after the first I/R challenge and may contribute to delayed preconditioning [22], superoxide dismutase (SOD) has received the most attention. In vivo (I/R) and in vitro (simulated I/R) studies indicate that SOD protein and activity is increased after the first challenge and interfering with SOD synthesis (antisense) prevents delayed preconditioning [23–26]. Similarly, both in vivo and in vitro studies indicate that NOS activity is increased after the first challenge and pharmacological inhibition of NOS prevents delayed preconditioning [15,21,27]. In addition, delayed preconditioning does not occur in iNOS deficient mice [28]. Since interfering with either SOD or NOS activity prevents delayed preconditioning, these two enzymes may not be independent effector systems, but interacting ones. The major objective of the present study was to assess the means by which these two enzyme systems interact in the development of delayed preconditioning. To this end, we used a reductionist approach [29], i.e., we exposed isolated cardiac myocytes to anoxia/reoxygenation (A/R; an in vitro correlate to I/R).

2. Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.1. Cells

Neonatal cardiac myocytes, adult myocardial endothelial cells, and adult PMN were isolated from wild-type mice (C57BL6) as previously described [16]. For some experiments neonatal cardiac myocytes or cardiac endothelial cells were isolated from iNOS or eNOS deficient mice (Jackson Laboratory). The eNOS and iNOS deficient mice were backcrossed for eight to 10 generations.

2.2. Anoxia/reoxygenation (A/R) protocol

The in vitro model of A/R used in the present study is similar to that described previously [15,16]. Briefly, confluent beating cardiac myocytes in 48-well plates were exposed to anoxia for 30 min in phenol red- and glucose-free DMEM and then reoxygenated (anoxia/reoxygenation, A/R). Anoxic conditions were obtained by equilibrating a small humidified plexiglass chamber containing the myocytes with 95% N_2 and 5% CO_2 via a catalytic deoxygenator apparatus (Engelhard Co., NJ, USA) and confirmed by measuring chamber pO_2. In this system, chamber pO_2 fell to 0 mmHg within 5 min after initiation of perfusion with the anoxic gas. Reoxygenation was achieved by exposing the cells to room air (CO_2 incubator). As a control, cardiac myocytes were exposed to normoxia (room air) rather than anoxia (normoxia/normoxia, N/N). The viability of cardiac myocytes is not affected by this A/R protocol [16] and this A/R model mimicks some of the key features of I/R models in vivo [17,30–32]. Among these shared features are: generation of inflammatory mediators by and activation of transcription factors in cardiac myocytes and endothelial cells, expression of adhesion molecules on endothelial cells, PMN adhesion to endothelial cells, and cardiac myocyte induced PMN transendothelial migration.

2.3. Oxidant production

Oxidant production within cardiac myocytes was assessed by measuring the oxidation of intracellular dihydrorhodamine 123 (DHR 123; Molecular Probes, Inc.), an oxidant-sensitive fluorochrome, as described previously [15,16]. Briefly, the cells were treated with DHR 123 (5 μM) for 1 h before being subjected to A/R or N/N. After A/R or N/N the cells were washed with PBS, lysed, and DHR 123 oxidation was assessed spectrofluorometrically at excitation and emission wavelengths of 502 and 523 nm, respectively.

2.4. PMN transendothelial migration

PMN transendothelial migration was assessed as previously described [15,33]. Briefly, mouse endothelial cells were grown to confluence on fibronectin-coated cell culture inserts (3-μm diameter pores). ^{51}Cr-labeled mouse

PMN in M199 were added to the apical aspect of the endothelial cell monolayers (PMN: endothelial cell ratio of 10:1) and co-incubated for 90 min with supernatants from A/R- or N/N-conditioned myocytes introduced into the basal compartment. The percentage of added neutrophils that migrated from the apical to the basal aspects of the insert membranes was quantified.

2.5. SOD activity and protein

Samples were obtained after the N/N or A/R protocols and total SOD activity was determined as previously described [16] using a Bioxytech SOD-525 kit (OXIS Health Products, Inc.) as recommended by the manufacturer. Mn-SOD activity was determined by subtracting the Cu/Zn-SOD activity obtained in chloroform:ethanol extracted samples (specifically inhibits Mn-SOD) from the total SOD activity. Mn-SOD protein in cardiac myocytes was assessed by Western blot as previously described [15]. Briefly, 50 μg of total cell lysates were resolved on 12.5% SDS–PAGE and transferred to polyvinylidene fluoride membranes (Osmonic, Inc.). Equal protein loading and transfer to the membranes was assured by staining the membranes with Ponceau-S. After blocking with 5% non-fat milk, the membranes were immunoblotted and the specific bands were visualized by BCIP/NBT staining and quantified by densitometry.

2.6. Antisense oligonucleotide

Mn-SOD antisense and sense oligodeoxyribonucleotides (ODN) were synthesized by ID Labs, Inc. The complete sequences for these ODN are described in Ref. [24]. This antisense ODN has previously been shown to prevent induction of Mn-SOD protein and activity in cardiac myocytes in vivo [23] and in vitro [24]. The antisense or sense ODN (5 μM) was applied to the myocytes 4 h before the initial A/R challenge and co-incubated for the entire experimental period (24 h).

2.7. Nitric oxide production

NO production by cardiac myocytes was assessed by measuring the fluorescence of 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate), a specific NO probe (Molecular Probes, Inc.). Briefly, DAF-FM diacetate (10 μM) in DMEM was added to the cardiac myocytes 1 h before the N/N or A/R challenge. After treatment, the myocytes and supernatants were collected and analyzed spectrofluorometrically at excitation and emission wavelengths of 495 and 515 nm, respectively.

2.8. RT-PCR

Total RNA was isolated from either N/N or A/R challenged cardiac myocytes with Trizal reagent and reverse transcribed into first strand cDNA by use of the Moloney murine leukemia virus reverse transcriptase system. The forward and reverse primers for eNOS were 5′-gatggcgaagcgtgtgaag-3′ and 5′-tcctccagccttgtgtcca-3′, respectively. The primer sequences for iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were the same as previously described [34]. The cDNAs were amplified within the linear range established for eNOS, iNOS, and GAPDH (35, 35 and 20 cycles, respectively).

2.9. Statistical analysis

All values are presented as mean±S.E.M. Statistical analysis was performed using ANOVA and Student's *t*-test (with a Bonferroni correction for multiple comparisons).

3. Results

3.1. In vitro model of delayed preconditioning

To characterize our in vitro model of delayed preconditioning, initial experiments were performed using cell death as the end point (propidium iodide). As shown in Fig. 1, treatment of myocytes with a 30 min A/R challenge did not result in myocyte death. However, an A/R challenge that involved a 1 h period of anoxia significantly increased cell death (N/N, A/R vs. N/N, N/N). Pretreatment of the myocytes with a 30 min A/R protocol significantly reduced myocyte death induced by the 1 h anoxic challenge (A/R, A/R vs. N/N, A/R). These findings confirm previous studies indicating that isolated cardiac myocytes can develop delayed preconditioning with respect to cytotoxicity [24–26]. If a 3 h period of anoxia was used during the A/R challenge (N/N, A/R) the increase in myocyte death was significantly greater than when a 1 h period of anoxia was used. Furthermore, there was no protection incurred by pretreatment with a 30 min A/R challenge. These latter findings indicate that if the A/R-induced myocyte injury is severe enough, a previous challenge with A/R may not result in the development of delayed preconditioning.

The focus of the present study was to assess the development of delayed preconditioning in cardiac myocytes with respect to a functional response, rather than cell death. To this end we assessed the development of a proinflammatory phenotype in cardiac myocytes in response to a 30 min A/R challenge; a model which does not induce myocyte death (Fig. 1). The endpoints targeted were (1) myocyte oxidant production and (2) the ability of myocytes to promote PMN transendothelial migration. As shown in Fig. 2A, the initial A/R challenge (N/N, A/R) significantly increased oxidant production within the myocytes as compared to the normoxic controls (N/N, N/N). The oxidant production induced by the second A/R

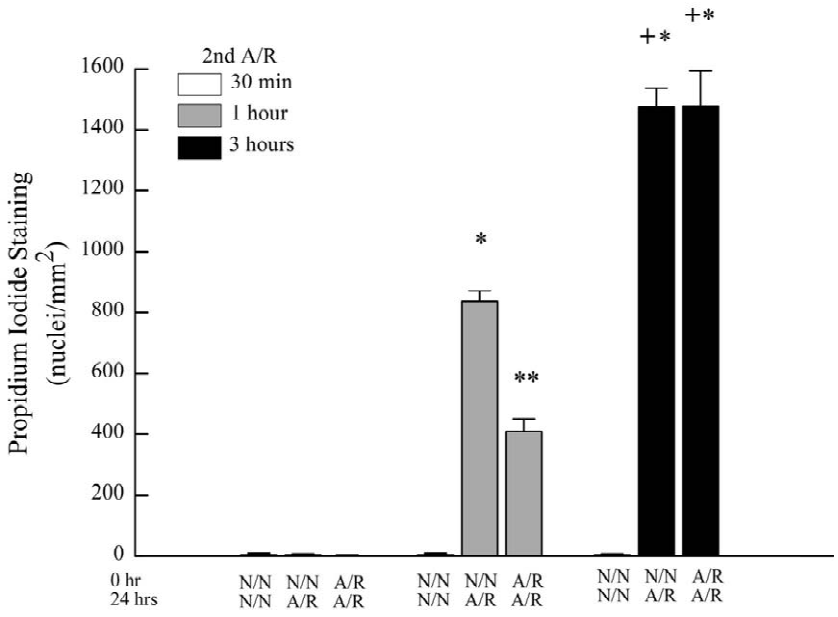

Fig. 1. Cardiac myocytes develop delayed preconditioning with respect to cell death. Propidium iodide (10 μM) was added to the cardiac myocytes after the second N/N or A/R challenges and the stained nuclei were counted 10 min later. The myocytes were pretreated with a 30 min A/R protocol and 24 h later exposed to a second A/R challenge (A/R, A/R) consisting of 30 min, 1 h, or 3 h anoxic periods. The initial A/R challenge provided protection against a subsequent A/R-induced cell death only when the second A/R challenge consisted of a 1 h period of anoxia. *$P<0.05$ compared to normoxic controls (N/N, N/N). **$P<0.05$ compared to N/N, A/R. +$P<0.05$ compared to an A/R protocol consisting of 1 h anoxia.

challenge (A/R, A/R) was significantly less than that induced by the initial A/R challenge (N/N, A/R). As shown in Fig. 2B, supernatants from A/R-conditioned myocytes (N/N, A/R) significantly increased PMN transendothelial migration when compared to supernatants from normoxic controls (N/N, N/N). The supernatants obtained after the second A/R challenge (A/R, A/R) induced significantly less PMN transendothelial migration than those obtained after the initial A/R challenge (N/N, A/R). Taken together, these findings indicate that the myocytes mounted an adaptive response after the first A/R challenge, which protected them from an oxidative stress and prevented them from generating chemotactic factors after the second A/R (delayed preconditioning). This delayed preconditioning could also be induced in cardiac myocytes by exposing the myocytes to H_2O_2, rather than an initial challenge with A/R.

3.2. Role of SOD in delayed preconditioning

The increase in oxidant production in myocytes during the initial A/R challenge appears to be causally linked to the ability of A/R-conditioned myocytes to promote PMN transendothelial migration ([16] and Fig. 2). In addition, previous studies indicate that SOD protein and activity are increased in myocytes by a hypoxic (pO_2 of 7 mmHg for 1 h) challenge [24]. Thus, we assessed whether induction of SOD by the initial A/R challenge in our model contributed to the development of delayed preconditioning. As shown in Fig. 3, during the first A/R challenge there were no significant changes in either Cu/Zn-SOD or Mn-SOD activity. However, 24 h later at the time of the second A/R challenge (A/R, A/R) the activity of Mn-SOD was significantly increased (Fig. 3B), while the activity of Cu/Zn-SOD was not significantly altered (Fig. 3A). As shown in the inset in Fig. 3B, the increase in Mn-SOD activity during the second A/R challenge (A/R, A/R) was associated with a significant increase in myocyte intracellular Mn-SOD protein (Western Blot) as compared to the initial A/R challenge (N/N, A/R).

Since Mn-SOD appeared to be the major form of SOD induced by the initial A/R challenge, we assessed whether induction of Mn-SOD played a role in the development of delayed preconditioning [24]. Myocytes were pretreated with an oligonucleotide containing an antisense sequence to the initiation site of mouse Mn-SOD for 4 h prior to the initial A/R challenge. This maneuver prevented the development of delayed preconditioning with respect to (1) myocyte oxidant production (Fig. 4A) and (2) the ability of myocytes to promote PMN transendothelial migration (Fig. 4B). As shown in Fig. 4A, A/R induced a significant increase in myocyte oxidant production; an effect not observed in myocytes pretreated with an A/R challenge 24 h earlier (compare A/R, A/R to N/N, A/R). In myocytes pretreated with the antisense Mn-SOD, the oxidant production during the second A/R challenge (A/R, A/R) was significantly greater than that noted in the absence of treatment with the antisense Mn-SOD (compare bars 3 and

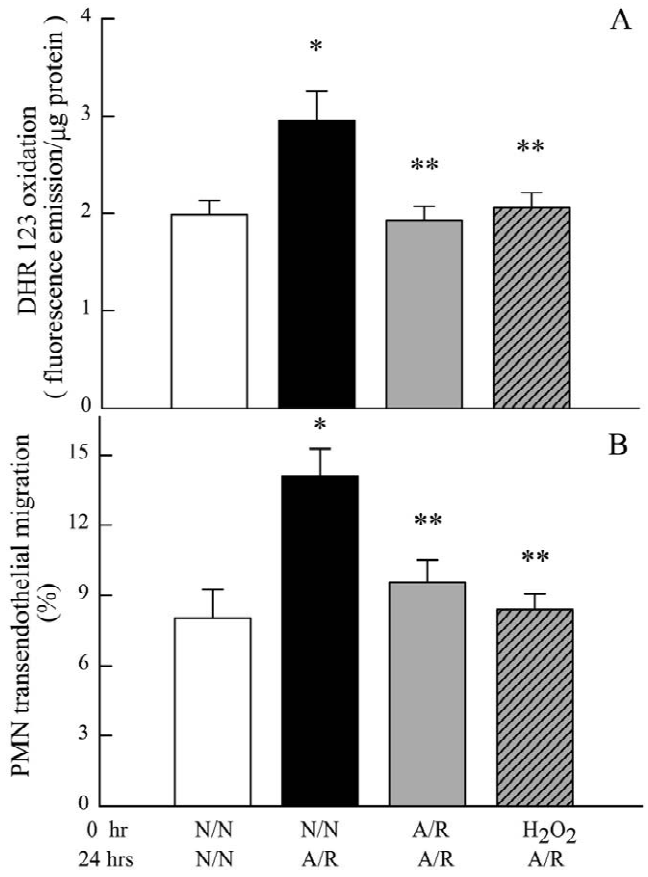

Fig. 2. Cardiac myocytes develop delayed preconditioning with respect to (A) intracellular oxidant production and (B) their ability to promote PMN transendothelial migration. Initially (0 h), cardiac myocytes were exposed to N/N, A/R, or H_2O_2 (12.5 μmol/l) and 24 h later challenged with either N/N or A/R. Myocyte oxidant production (A) and myocyte-induced PMN transendothelial migration (B) were increased when myocytes were exposed to A/R (N/N, A/R vs. N/N, N/N). These effects were significantly reduced when myocytes were preconditioned with either A/R (A/R, A/R) or H_2O_2 (H_2O_2, A/R). $n=4$, $^*P<0.05$, compared with N/N, N/N. $^{**}P<0.05$ compared to N/N, A/R.

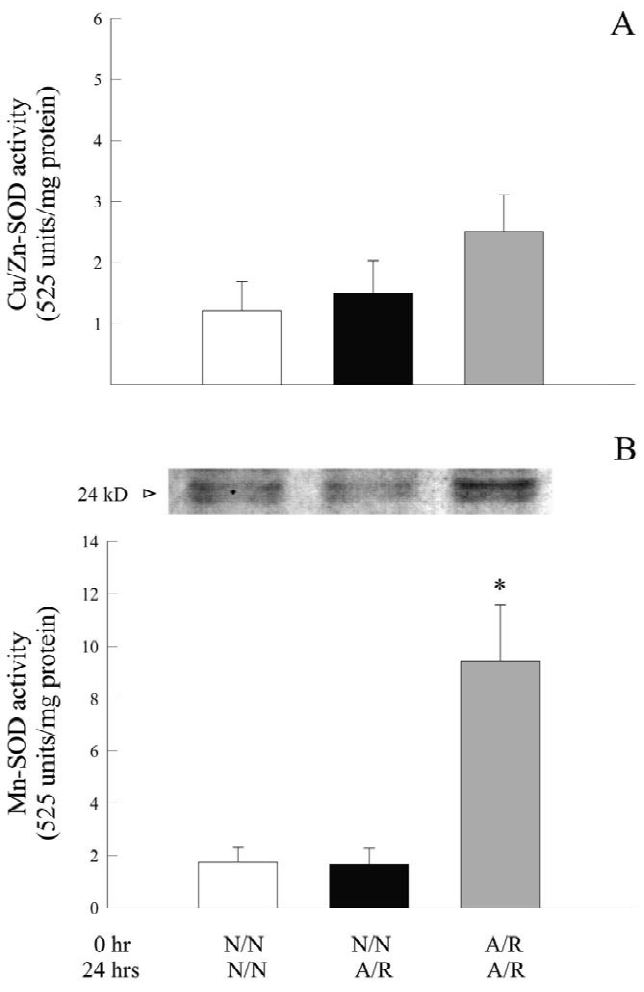

Fig. 3. Mn-SOD protein and activity are increased in cardiac myocytes 24 h after an A/R challenge. Initially (0 h), the myocytes were exposed to N/N or A/R and 24 h later challenged with either N/N or A/R and SOD protein and activity measured. (A) Cu/Zn-SOD activity was not significantly altered by any of the treatments. (B) Mn-SOD activity was significantly increased 24 h after the initial A/R challenge (A/R, A/R). $n=4$, $^*P<0.05$ as compared to N/N, A/R. (Inset) Mn-SOD protein (Western blot) in cardiac myocytes increased 24 h after the initial A/R challenge (A/R, A/R). Densitometric analysis of three blots indicated that there was a significant difference ($P<0.05$) between A/R, A/R and N/N, A/R (data not shown).

4). These observations indicate that induction of Mn-SOD plays an important role in the development of delayed preconditioning with respect to myocyte oxidant stress. Similarly, as shown in Fig. 4B, A/R-conditioned myocytes (N/N, A/R) significantly increased PMN transendothelial migration as compared to the normoxic controls (N/N, N/N). This A/R-induced increase in PMN transendothelial migration was significantly decreased when the myocytes were pretreated with an A/R challenge 24 h earlier (compare A/R, A/R to N/N, A/R). In myocytes pretreated with the antisense Mn-SOD, the PMN transendothelial migration induced by myocytes after the second A/R challenge (A/R, A/R) was significantly greater than that noted in the absence of treatment with the antisense Mn-SOD (compare bars 3 and 4). These findings indicate that induction of Mn-SOD plays an important role in the development of delayed preconditioning with respect to the ability of myocytes to promote PMN transendothelial migration.

3.3. Role of NO in delayed preconditioning

Cardiac myocytes contain both eNOS and iNOS isoforms [35,36]. Both of these isoforms have been implicated in the development of delayed preconditioning in the myocardium in vivo [21,27]. Thus, we assessed the role that these two isoforms of NOS play in the development of delayed preconditioning in our in vitro model. Our approach to this end was to utilize cardiac myocytes isolated from iNOS and eNOS deficient mice in the A/R protocols.

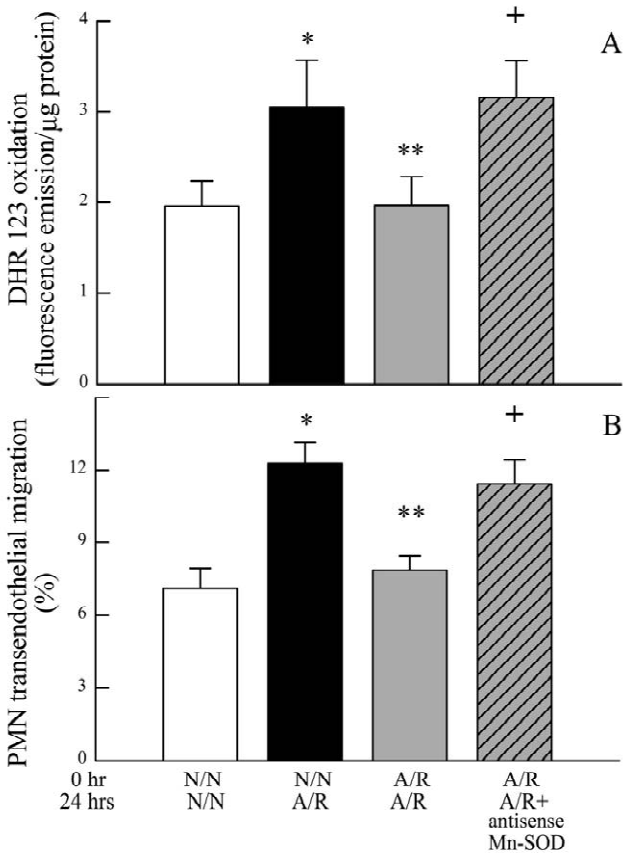

Fig. 4. Induction of Mn-SOD is required for the development of delayed preconditioning in cardiac myocytes with respect to oxidant production (A) and PMN transendothelial migration (B). The first three bars represent the development of delayed preconditioning similar to the data presented in Fig. 2. The antisense oligonucleotide prevented the development of delayed preconditioning, i.e., the increase in myocyte oxidant production (A) and the increase in PMN transendothelial migration (B) were significantly greater than in myocytes not pretreated with the Mn-SOD antisense (A/R, A/R with antisense Mn-SOD vs. A/R, A/R). The sense Mn-SOD was without effect (data not shown). $n=6$ for oxidant production and $n=3$ for PMN transendothelial migration. *$P<0.05$ as compared to N/N, N/N. **$P<0.05$ as compared to N/N, A/R. +$P<0.05$ as compared to A/R, A/R.

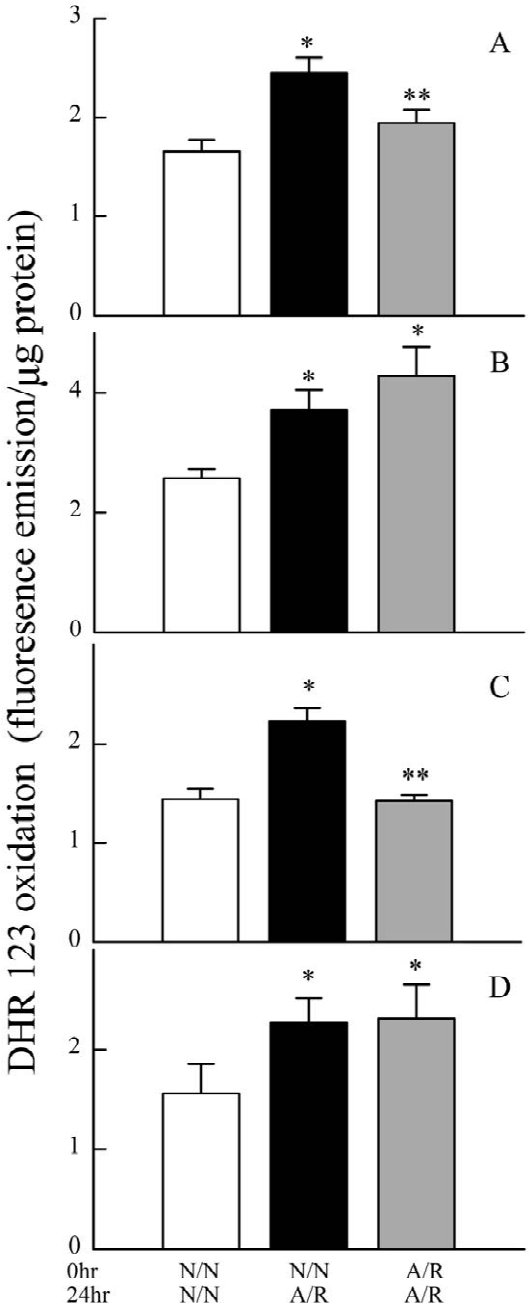

Fig. 5. The development of delayed preconditioning with respect to myocyte oxidant production is dependent on eNOS, but not iNOS. (A) In cardiac myocytes derived from iNOS deficient mice, the development of delayed preconditioning with respect to oxidant production was similar to that observed in wild-type mice (compare to Fig. 2A). (B) In cardiac myocytes derived from eNOS deficient mice there was no measurable evidence for the development of delayed preconditioning, i.e., the oxidant production incurred after the second A/R challenge was also significantly increased (compare N/N, A/R to A/R, A/R). Nearly identical results were obtained with cardiac endothelial cells derived from iNOS (C) and eNOS (D) deficient mice. $n=4$. *$P<0.05$ as compared to N/N, N/N. **$P<0.05$ as compared to N/N, A/R.

For these studies we focused on myocyte oxidant production during the development of delayed preconditioning, since this appears to be the initiating factor [15,16,26]. As shown in Fig. 5A, the initial A/R challenge of myocytes (N/N, A/R) derived from iNOS deficient mice resulted in a significant increase in myocyte DHR oxidation indicative of an increase in myocyte oxidant production (N/N, A/R vs. N/N, N/N). If the myocytes were pretreated with an A/R challenge 24 h earlier (A/R, A/R), the second A/R challenge induced less DHR oxidation compared to the non-preconditioned protocol (N/N, A/R), thus demonstrating less oxidant production (delayed preconditioning). In a similar fashion, the initial challenge of myocytes derived from eNOS deficient animals resulted in a significant increase in myocyte oxidant production (Fig. 5B; N/N, A/R vs. N/N, N/N). However, an A/R challenge imposed 24 h after an initial A/R challenge (A/R, A/R) to myocytes from eNOS deficient mice still resulted in a significant increase in myocyte oxidant

production (no delayed preconditioning). Nearly identical results were obtained when delayed preconditioning was assessed in isolated cardiac endothelials from iNOS and eNOS deficient mice (Fig. 5C and D, respectively).

The effects of A/R on transcription of iNOS and eNOS in cardiac myocytes derived from wild-type mice are shown in Fig. 6. In myocytes derived from wild-type mice, mRNA for eNOS, but not iNOS, increased after the initial A/R challenge. The eNOS mRNA levels significantly increased by 4 h after the initial A/R challenge and returned toward control levels thereafter.

As shown in Fig. 7A, cardiac myocytes isolated from wild-type mice produced significantly more NO during the second A/R challenge (A/R, A/R) as compared to the initial A/R challenge (N/N, A/R). As shown in Fig. 7B, myocytes derived from eNOS deficient mice did not significantly alter their NO production during the A/R challenges. Fig. 8 illustrates the effects of L-NAME on the development of delayed preconditioning in cardiac myocytes (from wild-type mice) with respect to oxidant production. L-NAME was added prior to the second challenges with either N/N or A/R. In the presence of L-NAME the initial A/R challenge (N/N, A/R) and the second A/R challenge (A/R, A/R) significantly increased oxidant production within the myocytes as compared to the normoxic controls (N/N, N/N). These findings indicate that, in the presence of the NOS inhibitor L-NAME, myocytes do not develop delayed preconditioning with respect to oxidant production (compare to Fig. 2A).

3.4. Interaction between SOD and NOS in delayed preconditioning

Previous studies have indicated that superoxide and NO can interact to result in the formation of products with biologic activity [37]. Thus, we assessed Mn-SOD activity during the development of delayed preconditioning in myocytes derived from iNOS and eNOS deficient mice. As shown in Fig. 9A, Mn-SOD activity in myocytes derived from iNOS deficient mice was significantly increased during the second A/R challenge, much like the situation in wild-type mice (see Fig. 3B). The situation was much different in myocytes derived from eNOS deficient mice, i.e., Mn-SOD activity was not significantly altered during the second A/R challenge. Mn-SOD protein levels during the development of delayed preconditioning in myocytes

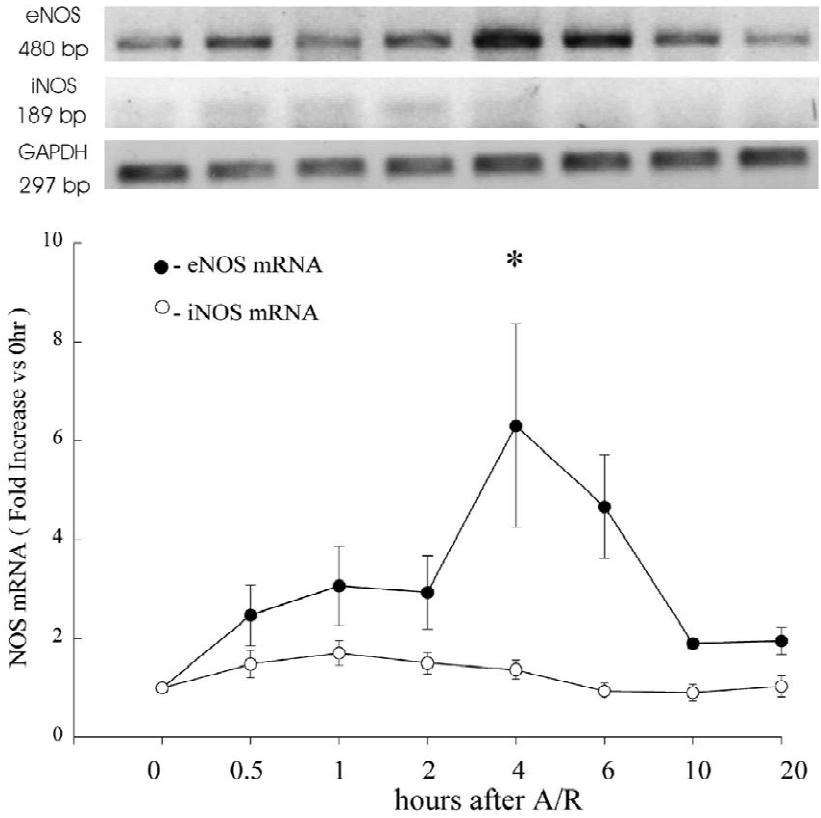

Fig. 6. Message levels for eNOS, but not iNOS, are significantly increased in cardiac myocytes derived from wild-type mice challenged with A/R. The myocytes were challenged with A/R and then processed at different times thereafter to evaluate their levels of mRNA for eNOS and iNOS by RT-PCR. A representative experiment is shown in the upper panel and the results of three experiments are shown in the lower panel. Densitometric analyses of autoradiographs for quantitation of eNOS and iNOS mRNAs were normalized to GAPDH mRNA. The eNOS mRNA levels significantly increased by 4 h after the A/R challenge and returned toward control levels thereafter. By contrast, iNOS mRNA levels were not affected by the A/R challenge. ANOVA and *t*-test, $*P<0.05$.

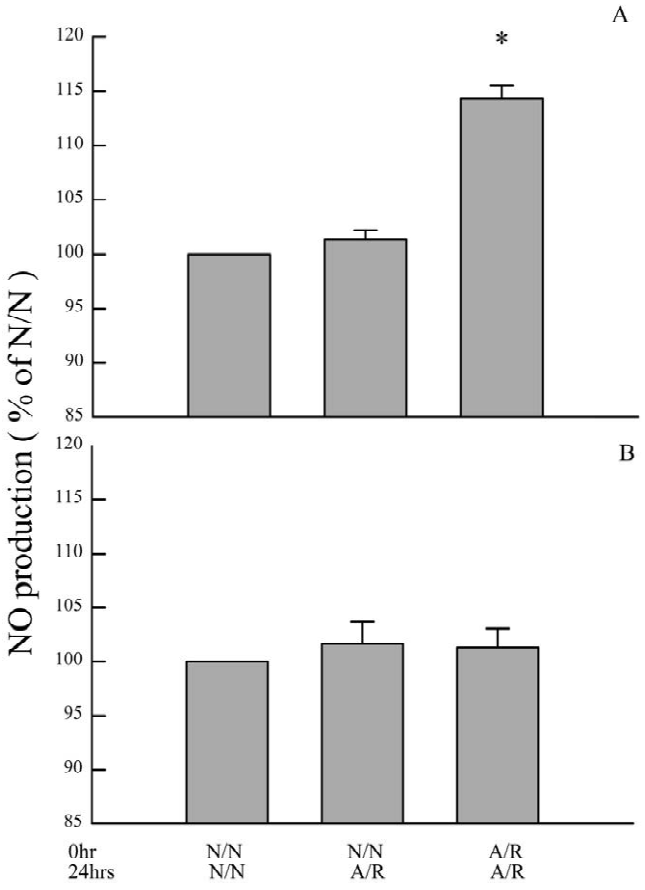

Fig. 7. Nitric oxide (NO) production by cardiac myocytes from wild-type mice (A), but not eNOS deficient mice (B), is significantly increased during the second A/R challenge. Data shown are fluorescence emission in supernatants (400 μl); intracellular fluorescence emission was not significantly affected by treatments. Data are expressed as percent of normoxic controls (N/N, N/N). The normoxic control values for NO production (fluorescence emission/μg protein) by wild-type and eNOS deficient myocytes were 13.7±0.9 and 5.3±1.2, respectively. $n=3$. *$P<0.05$ as compared to N/N, A/R.

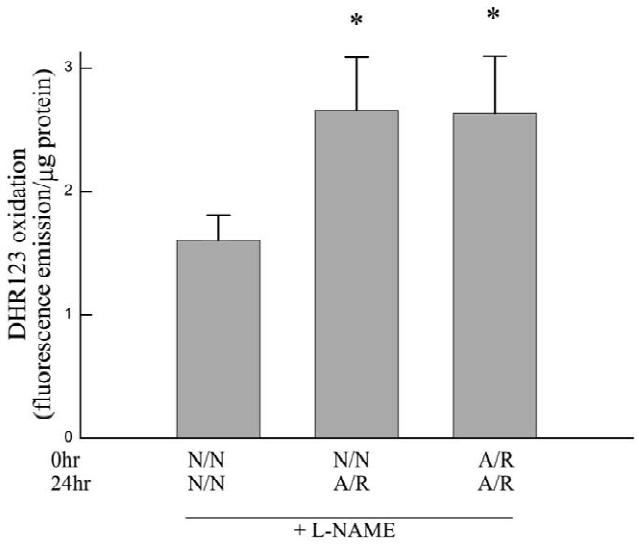

Fig. 8. The NOS inhibitor, L-NAME, prevents the development of delayed preconditioning in cardiac myocytes, with respect to oxidant production. The experimental protocol was the same as described in Fig. 2. L-NAME (100 μM) was added to the myocytes 1 h prior to the second challenge with N/N or A/R. Oxidant production was significantly increased both after the initial A/R challenge (N/N, A/R) and second A/R challenge (A/R, A/R). $n=3$. *$P<0.05$ as compared to N/N, N/N.

derived from eNOS and iNOS deficient mice are shown in Fig. 9B. In myocytes derived from both eNOS and iNOS deficient mice, Mn-SOD protein was significantly increased during the second A/R challenge (A/R, A/R vs. N/N, A/R).

4. Discussion

In the present study we used a reductionist approach to provide evidence that isolated cardiac myocytes can develop delayed preconditioning with respect to the ability of an A/R challenge to induce a proinflammatory phenotype. A/R-conditioned myocytes can promote PMN transendothelial migration (Fig. 2B). However, if the myocytes are pretreated with an A/R challenge, a subsequent A/R challenge imposed 24 h later does not result in an induction of a proinflammatory phenotype, i.e., the myocytes promote less PMN transendothelial migration than that observed after the first A/R challenge (Fig. 2B). Previous studies have implicated both superoxide and nitric oxide in delayed preconditioning [4,18,19,21]. In the present study, we provide evidence that may reconcile these apparently opposing views; i.e., that NOS and SOD enzymes may act in concert during the development of delayed preconditioning.

The ability of A/R-conditioned myocytes to promote PMN transendothelial migration is intimately linked to the A/R-induced intracellular oxidant production ([16] and Fig. 2A). The development of delayed preconditioning (with respect to PMN transendothelial migration) is associated with a limited intracellular oxidant stress in myocytes during the second A/R challenge (Fig. 2A). Previous in vitro studies using approaches to simulate I/R indicate that the endogenous antioxidant enzyme, SOD, is induced after the first challenge and contributes to the protection from cell death during the second challenge [24,25]. Although we used a functional index (proinflammatory phenotype) rather than cell death, our findings are in general agreement with these previous studies. We show that Mn-SOD protein and activity are increased during the second A/R challenge (Fig. 3). Furthermore, prevention of the induction of Mn-SOD (antisense) by the first A/R challenge results in an attenuation of the development of delayed preconditioning with respect to (1) myocyte oxidant production (Fig. 4A) and (2) the ability of myocytes to promote PMN transendothelial migration (Fig. 4B). Taken together, these observations provide compelling support

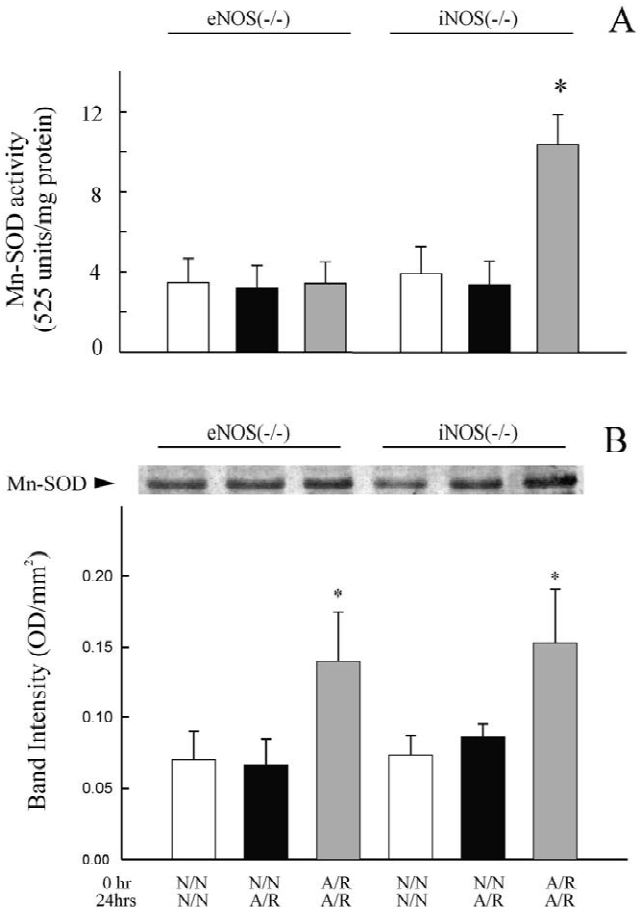

Fig. 9. During the second A/R challenge, Mn-SOD protein is increased in cardiac myocytes derived from both iNOS and eNOS deficient mice, while Mn-SOD activity is increased only in myocytes derived from iNOS mice. The experimental protocol was the same as described in Fig. 3. (A). In cardiac myocytes derived from iNOS deficient mice (iNOS−/−), Mn-SOD activity was significantly increased after the second A/R challenge (compare A/R, A/R to N/N, A/R). In cardiac myocytes derived from eNOS deficient mice (eNOS−/−), Mn-SOD activity was not significantly altered after the second A/R challenge (compare A/R, A/R to N/N, A/R). (B) The bands obtained from a representative Western blot are shown above and quantitation of the results of three blots (densitrometry) are shown below (histograms). Mn-SOD protein levels were significantly increased during A/R, A/R in cardiac myocytes derived from either eNOS or iNOS deficient mice. $n=3$ for both (A) and (B). *$P<0.05$ as compared to N/N, A/R.

for the contention that Mn-SOD is an important effector enzyme in the development of delayed preconditioning in isolated cardiac myocytes.

Previous studies also indicate that NOS plays an important role in the development of delayed preconditioning [15,27,38]. Indeed, NOS has been implicated as both the initiating molecule (eNOS-derived) and the effector molecule (iNOS-derived). In the present study, we provide additional support for the contention that NOS plays an important role in delayed preconditioning. However, only one isoform of NOS appears to be involved in delayed preconditioning in our experimental model. As shown in Fig. 5A, delayed preconditioning (with respect to the A/R-induced intracellular oxidant production) occurred in myocytes isolated from iNOS deficient mice. By contrast, there was no measurable evidence for delayed preconditioning (with respect to oxidant production) in myocytes isolated from eNOS deficient mice (Fig. 5B). In addition, when we assessed changes in iNOS and eNOS messages after an A/R challenge to myocytes derived from wild-type mice, only eNOS mRNA was increased (Fig. 6). Myocytes isolated from wild-type mice, but not from eNOS deficient mice, produced and released NO into the surrounding milieu (Fig. 7). Finally, L-NAME, a NOS inhibitor, prevented the development of delayed preconditioning with respect to myocytes oxidant production (compare Figs. 8 and 2A). Taken together, these observations indicate that eNOS is involved in the development of delayed preconditioning.

Our findings that myocytes derived from iNOS deficient animals developed delayed preconditioning are in contrast with previous in vivo studies, which indicate that delayed preconditioning does not occur in the myocardium of iNOS deficient mice [28]. An exact explanation for these discordant findings is not readily apparent, but two possibilities appear plausible. One possibility is that the end points measured were different. In the in vivo studies, myocardial infarction (or cell death) was a major endpoint. By contrast, in the present study cell death was not an end point, rather a functional aspect (proinflammatory phenotype) was assessed. Thus, in delayed preconditioning the mediators involved in preventing cell death may be quite different from those involved in limiting parenchymal cell dysfunction. Another possibility is that multiple signals from different resident cells in the myocardium contribute to the end result in vivo [39]. To address the latter possibility, we evaluated the development of delayed preconditioning (with respect to oxidant production) in cardiac endothelial cells isolated from iNOS and eNOS deficient mice. As was the case with isolated cardiac myocytes, isolated cardiac endothelial cells from iNOS deficient mice developed delayed preconditioning, while endothelial cells from eNOS deficient mice did not (Fig. 5C and D, respectively). Thus, when isolated from their in vivo milieu, both cardiac myocytes and endothelial cells rely on eNOS-derived NO to develop delayed preconditioning. In addition, previous in vitro studies indicate that exposing rat cardiac myocytes to hypoxia results in only a modest increase in iNOS mRNA [40]. However, if the cardiac myocytes are exposed to hypoxia in combination with a cytokine there is a substantial amount of iNOS mRNA generated. Thus, in vivo, other cell types within the myocardium (e.g., mast cells or fibroblasts) may be generating cytokines that would result in an induction of iNOS in myocytes or endothelial cells. This latter possibility is supported by the finding that mRNA for cytokines can be detected in the myocardium after an I/R challenge in vivo [41]. Irrespective of the explanation for the

discordant results between in vivo and in vitro studies, the results of the present study clearly indicate that eNOS-derived NO is a prerequisite for the development of delayed preconditioning in isolated cardiac myocytes.

Our findings indicate that both eNOS and Mn-SOD appear to be critical effector enzymes in the development of delayed preconditioning in cardiac myocytes. These two enzyme systems may be acting independently or, alternatively, they may be acting in concert in this phenomenon. In the present study, we assessed whether there was a potential interaction between these two enzymes in the development of delayed preconditioning. Our approach to address this issue was to examine changes in Mn-SOD activity and protein during the development of delayed preconditioning in myocytes derived from iNOS and eNOS deficient mice. As shown in Fig. 9A, in myocytes derived from iNOS deficient mice (which developed delayed preconditioning) Mn-SOD activity was increased during the second A/R challenge. By contrast, in myocytes derived from eNOS deficient mice (which did not develop delayed preconditioning) Mn-SOD activity was not significantly altered. Furthermore, in myocytes derived from either iNOS or eNOS deficient mice, Mn-SOD protein was increased to the same extent during the second A/R challenge (Fig. 9B). Collectively, these observations indicate that NO derived from eNOS is not affecting Mn-SOD transcription, but rather is modulating the activity of the enzyme; e.g., limiting signals which inhibit its activity.

The present study focused on the role of Mn-SOD and eNOS in delayed preconditioning with respect to a proinflammatory phenotype in isolated cardiac myocytes. This approach was based on the existing evidence that PMN infiltration is an important component of the myocardial injury incurred after I/R [1,8–14]. Given that the classical view of delayed preconditioning involves protection against I/R-induced myocardial infarction (necrosis), the relevance of our approach to the in vivo situation deserves comment. As mentioned previously, different mechanisms may be involved in the development of delayed preconditioning in vivo, e.g., iNOS [27,28]. Thus, since we used isolated myocytes, the impact of other resident myocardial cells and/or circulating factors on this phenomenon was excluded. Nonetheless, the use of isolated cells allows for the delineation of the specific role of the cell under study in the development of delayed preconditioning [29]. In addition, we used a functional endpoint (inflammation), rather than cell death, to assess mechanisms involved in the development of delayed preconditioning. Interestingly, in vitro models using isolated myocytes have also demonstrated a role for Mn-SOD in the protection from death afforded by delayed preconditioning [24]. Thus, our approach has uncovered mechanistic details that appear to be similar to in vitro models in which cell death was used as an endpoint. Nonetheless, our findings using isolated cardiac myocytes and a functional endpoint (inflammation) should be interpreted in the light of the above-mentioned caveats.

In summary, our findings indicate that the enzymes eNOS and Mn-SOD may work in concert to ensure the development of delayed preconditioning. During the first A/R challenge an oxidant production is generated in cardiac myocytes. This oxidant production transforms the myocytes into a pro-inflammatory phenotype, i.e., capable of generating a chemotactic gradient and promoting PMN transendothelial migration. In addition, the oxidant production sets into motion a series of events (e.g., nuclear transcription factors) that result in the induction of Mn-SOD and eNOS. We have preliminary evidence implicating NFκB in transcription of Mn-SOD [31] and AP-1 in transcription of eNOS in our model (unpublished observations). The resultant increase in Mn-SOD activity serves to protect the cardiac myocytes from the oxidant stress imposed by the second A/R challenge. In addition, the increase in eNOS activity modulates Mn-SOD activity by preventing its inactivation.

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