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Therapeutic delivery of hydrogen sulfide for salvage of ischemic skeletal muscle after the onset of critical ischemia

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Background: Recent evidence suggests that hydrogen sulfide is capable of mitigating the degree of cellular damage associated with ischemia-reperfusion injury (IRI).

Methods: This study evaluated the potential utility of hydrogen sulfide in preventing IRI in skeletal muscle by using in vitro (cultured myotubes subjected to sequential hypoxia and normoxia) and in vivo (mouse hind limb ischemia, followed by reperfusion) models to determine whether intravenous hydrogen sulfide delivered after the ischemic event had occurred (pharmacologic postconditioning) conferred protection against IRI. Injury score and apoptotic index were determined by analysis of specimens stained with hematoxylin and eosin and terminal deoxynucleotide transferase-mediated deoxy-uridine triphosphate nick-end labeling, respectively.

Results: In vitro, hydrogen sulfide reduced the apoptotic index after 1, 3, or 5 hours of hypoxia by as much as 75% (*P* = .002), 80% (*P* = .006), and 83% (*P* < .001), respectively. In vivo, hydrogen sulfide delivered after the onset of hind limb ischemia and before reperfusion resulted in protection against IRI-induced cellular changes, which was validated by significant decreases in the injury score and apoptotic index. The timing of hydrogen sulfide delivery was crucial: when delivered 20 minutes before reperfusion, hydrogen sulfide conferred significant cytoprotection (*P* < .001), but treatment 1 minute before reperfusion did not provide protection (*P* = NS).

Conclusions: These findings confirm that hydrogen sulfide limits IRI-induced cellular damage in myotubes and skeletal muscle, even when delivered after the onset of ischemia in this murine model. These data suggest that when given in the appropriate dose and within the proper time frame, hydrogen sulfide may have significant therapeutic applications in multiple clinical scenarios. (J Vasc Surg 2011;53:785-91.)

Clinical Relevance: Extremity ischemia is a limb- and life-threatening clinical scenario that must be treated expeditiously. Although rapid recognition and revascularization is crucial, there are few options regarding pharmacologic means to mitigate the effect of ischemia and subsequent reperfusion (ischemia-reperfusion injury). This is particularly true in situations of unanticipated ischemia, where no interventions can be undertaken before the onset of ischemia. Hydrogen sulfide is protective of muscle cells against ischemia-reperfusion injury when delivered before the onset of ischemia, and this study evaluated the effect of hydrogen sulfide delivered after the onset of ischemia. The promising results of the in vitro and in vivo studies suggest that hydrogen sulfide may in the future be an important adjunct to prompt surgical or endovascular intervention in episodes of unanticipated extremity ischemia.

Acute vascular occlusion of the lower extremity can result from embolism, thrombosis, or extrinsic compression. Because of the relatively high metabolic rate of even resting muscle tissue, an ischemic interval as short as 2

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hours may begin to produce severe and irreversible injury to myocytes. This is likely caused by the initial stress of the ischemia, which is further exacerbated by the generation of large quantities of reactive oxygen species upon reperfusion and exposure of the ischemic tissue to oxygen-containing blood.

The damage caused upon revascularization of acutely ischemic tissue, known as ischemia-reperfusion injury (IRI), ranges from edema to frank necrosis, depending upon the length of the ischemic interval and the tissue(s) involved. In the lower extremity, obligatory swelling caused by reperfusion may result in further ischemia because of the limited compliance of the thick fascial compartments of the lower leg. To prevent further compromise of the neural and muscle tissues within the compartments, fasciotomies must be performed, often leading to immobilization, skin grafting, and other morbidity due to the prolonged recovery period.

For scenarios of planned ischemia, such as organ transplantation and free tissue transfer, strategies have been devised to protect tissue from the sequelae of IRI. Theo-

retically, a cytoprotective agent delivered before the onset of ischemia could ameliorate the consequences of IRI. Toward that end, several compounds have been evaluated, with varying degrees of success.[1-3]

In the clinical setting, however, the onset of acute ischemia is often unanticipated, which makes the delivery of a protective agent before the onset of ischemia impossible. Thus, for a compound to be efficacious, it must provide protection when given *after* the onset of ischemia.

Hydrogen sulfide (HS) has historically been thought of as an environmental toxic gas,[4-6] but it is now known to be an endogenous signaling molecule[7-11] in the gasotransmitter family, along with nitric oxide and carbon monoxide.[12-14] This molecule provides significant protection in cardiac, neurologic, and hepatic models of IRI when delivered before an ischemic insult.[15-22]

Our laboratory recently demonstrated that pharmacologic preconditioning with HS confers significant long-term protection against IRI in skeletal muscle for ischemic intervals lasting up to 3 hours.[23] The potential cytoprotective effect of HS in skeletal muscle when delivered after the onset of ischemia, however, has not yet been broadly evaluated. Furthermore, none of the previous studies fully examined the relationship between the timing of HS delivery and its protective efficacy. Therefore, the purpose of this study was to determine whether pharmacologic postconditioning with HS is efficacious in reducing IRI in skeletal muscle.

METHODS

HS donor modalities. For in vitro as well as in vivo studies, the HS donor was NaHS (Alfa Aesar, Ward Hill, Mass) dissolved in sterile phosphate buffered saline (PBS; EMD Chemicals; Gibbstown, NJ), with a final pH of 8.5.

In vitro myotube anoxia-normoxia studies. Murine myoblasts (ATCC, Manassas, Va) were grown in culture at 37°C in Dulbecco's Modified Eagle's Media (Mediatech, Manassas, Va) that had been supplemented with 10% fetal bovine serum (JR Scientific, Woodland, Calif) and 1% penicillin/streptomycin (Mediatech) until the cells became 80% confluent. Myoblasts were then were plated into 8-well chamber slides (Nunc, Rochester, NY) at a density of 1×10^4 cells/well and subsequently differentiated into myotubes upon exposure to media containing 2% horse serum (PML Microbiologicals, Wilsonville, Ore) in place of the fetal bovine serum. All studies were performed on passage 4 cells, 4 days after exposure to horse serum.

The cells were exposed to 1, 3, or 5 hours of hypoxia (1% oxygen) in a dual-chamber hypoxia chamber. Cells were treated 20 minutes before the ends of the hypoxic period with the same horse serum-containing media, modified by the addition of equal volumes of varying concentrations of NaHS to bring the final HS concentration in the media to 0, 0.1, 1.0, 10, 100, and 1000 μM. The pH of the media (8.5) did not change with addition of NaHS. At the end of the hypoxic period, the cells were returned to a standard incubator for 3 hours of normoxia (21% oxygen). A separate sample of myotubes plated in the same manner

and treated with the same amount of NaHS-containing media was kept at 21% oxygen as a normoxic control. Myotubes were then fixed in 4% para-formaldehyde (Alfa Aesar) for 1 hour and stored at −20°C. All experiments were repeated in triplicate.

Assay to assess apoptosis. The terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was used to assess the degree of apoptosis. The in situ cell death detection kit (Roche Applied Science, Indianapolis, Ind) with Prolong Gold Antifade reagent and 4′,6-diamidino-2-phenylindole (DAPI) counterstain (Invitrogen, Carlsbad, Calif) were used per the manufacturers' instructions. Briefly, slides were rinsed in PBS for 5 minutes and permeabilized with 0.1% Triton X-100 (MP Biomedicals, Solon, Ohio) and 0.1% citric acid (Mallinckrodt, Hazelwood, Mo). The TUNEL solution was applied to all slides for 1 hour, and then the counterstain was applied and allowed to develop overnight.

Determination of apoptotic index. TUNEL-stained myotubes were analyzed using florescent microscopy. The apoptotic index (AI) was determined by quantifying the average number of TUNEL-positive myocyte nuclei divided by the number of TUNEL-negative/DAPI-positive myocyte nuclei across five random high-powered fields (original magnification, ×200) by two blinded observers. Fluorescent microscopy was performed with a Nikon e800 upright microscope with DAPI/fluorescein isothiocyanate filter (Nikon, Tokyo, Japan). Values are presented as mean ± standard error of the mean.

In vivo mice studies. All in vivo studies used 8-week-old male C57Bl/6 mice (Jackson Laboratory, Bar Harbor, Me). All animal care and experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals[24] and were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee (IACUC # 0704-607A).

Murine hind limb ischemia-reperfusion studies. Eighteen mice were anesthetized by an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (60 mg/kg). Hind limb ischemia was performed as previously described.[23] Briefly, a tourniquet was applied around the hind limb, superior to the greater trochanter, thereby occluding all blood flow to distal tissue. The contralateral leg served as a nonischemic control. Either 1 or 20 minutes before the end of the ischemic period, mice received a tail vein injection of 200 μL PBS containing 0 or 0.98 μg NaHS. Based on the estimated circulating blood volume (7% of 25 g, or 1.75 mL), this amount of NaHS raised the final bloodstream concentration to 10 μM ($n = 6$ for each group). Fig 1 summarizes the timeline for HS delivery for all experiments.

After 3 hours of ischemia, the tourniquet was removed and the ischemic limb was allowed to reperfuse for 3 hours (reperfusion was confirmed by the rapid onset of hyperemia of the limb). At that time, the mice were euthanized by carbon dioxide asphyxiation and cervical dislocation ac-
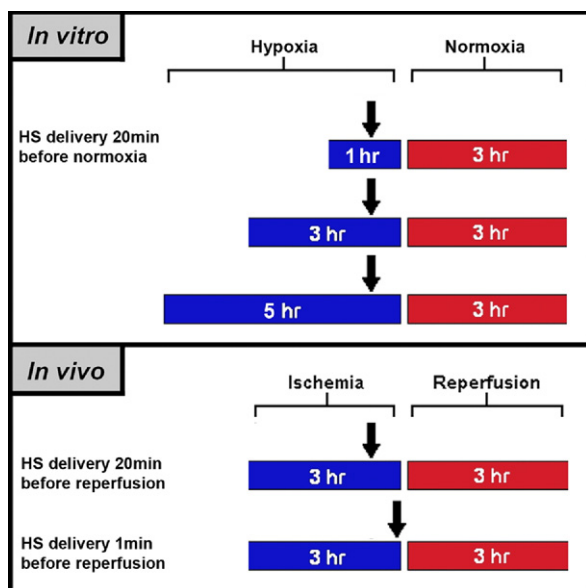
Fig 1. Summary of time of NaHS delivery *(arrow)* for the pre-ischemic, preischemic (long-term), postischemic (1 minutes before reperfusion), and postischemic (20 minutes before reperfusion) experimental groups.

cording to institutional protocol, and the gastrocnemius muscle was harvested and processed.

Tissue processing. Excised muscle was washed in PBS and dissected free of excess fat and fascia and fixed in 10% buffered formalin for 24 hours, dehydrated, then embedded in paraffin. Ten-micrometer sections of all samples were stained with hematoxylin and eosin (H&E) or TUNEL (staining and apoptotic index calculation were performed as described above).[25]

Determination of injury score. The injury score (IS) was determined by a protocol based on that which has been established by McCormack et al.[26] Four random photomicrographs were taken of each H&E-stained section, and based on the proportion of injured cells (defined by ragged cellular borders, vacuolization, lymphocytic infiltration, or rhabdomyolysis), blinded reviewers assigned a numerical value between 0 and 10 for each in vivo experiment. Values are presented as mean ± standard error of the mean.

Statistical analysis. Statistical significance was determined by first performing a square root transformation to the data to correct for heteroscedasticity (differing variances). Subsequently, a series of independent measures t-tests with a family error rate of $\alpha = 0.05$ was performed.

RESULTS

In vitro study. In myotubes subjected to 1 hour of hypoxia, followed by 3 hours of normoxia, non-HS-treated cells had a mean AI of 5.7% ± 1.0%, cells treated with 1 μM HS had a mean AI of 2.6% ± 0.3% ($P = .013$), cells treated with 10 μM HS had a mean AI of 1.0% ± 0.3% ($P = .002$), and cells treated with 100 μM had a mean AI of 1.4% ±

0.5% ($P = .006$; Fig 2). In myotubes subjected to 3 hours of hypoxia, followed by 3 hours of normoxia, non-HS-treated cells had a mean AI of 8.4% ± 2.2%, cells treated with 1 μM HS had a mean AI of 2.1% ± 0.7% ($P = .016$), cells treated with 10 μM HS had a mean AI of 1.7% ± 0.3% ($P = .006$), and cells treated with 100 μM had a mean AI of 3.4% ± 0.5% ($P = $ NS). In myotubes subjected to 5 hours of hypoxia, followed by 3 hours of normoxia, non-HS-treated cells had a mean AI of 8.9% ± 1.0%, cells treated with 1 μM HS had a mean AI of 1.5% ± 0.3% ($P < .001$), cells treated with 10 μM HS had a mean AI of 1.7% ± 0.4% ($P < .001$), and cells treated with 100 μM had a mean AI of 3.0% ± 0.3% ($P < .001$).

In HS-treated normoxic cells, no difference in the rate of apoptosis compared with control (non-HS treated, normoxic) was noted at any of the tested doses (data not shown).

In vivo studies. In vivo studies in which NaHS was delivered after the induction of ischemia, but before reperfusion, demonstrated a relative preservation of normal muscle architecture with minimal extracellular edema when delivered 20 minutes before reperfusion. In contrast, this protection was not observed when NaHS was delivered only 1 minute before reperfusion, which resulted in significant membranous discontinuity, extracellular edema, and intracellular vacuolization (Fig 3). The IS was 3.4 ± 0.8 for muscle from non-NaHS-treated mice, 2.1 ± 0.2 ($P = $ NS) for those treated with 10 μM NaHS 1 minute before reperfusion, and 0.5 ± 0.1 ($P < .001$) for those treated with 10 μM NaHS 20 minutes before reperfusion. The TUNEL assay showed that at the 10 μM dose, the AI was reduced by 84% ($P < .001$) when delivered 20 minutes before reperfusion and was reduced by only 29% ($P = $ NS) when delivered 1 minute before reperfusion (Fig 4).

DISCUSSION

Unanticipated acute ischemia remains a challenging clinical problem. In tissues with a high metabolic demand, irreversible damage may occur when perfusion is re-established after even brief ischemic intervals, the result of an abundance of reactive oxygen species generated from the sudden influx of oxygen-bearing blood. Because of the relatively high metabolic rate of skeletal muscle (even at rest), the window for successful revascularization of the acutely ischemic lower extremity remains brief.

Even when revascularization occurs in a relatively timely fashion, subsequent IRI often results in cellular and tissue edema that may necessitate further morbid interventions such as fasciotomy. When profound ischemia lasts >2 hours, irreversible damage occurs to muscle and nerve tissue within the affected limb. In the short term, myonecrosis may lead to myoglobinuria and renal failure. Longer-term sequelae include fibrosis and contracture of the affected muscle, which in combination with ischemic nerve damage results in permanent and critical disability. In more severe cases, gangrene and subsequent amputation may result.
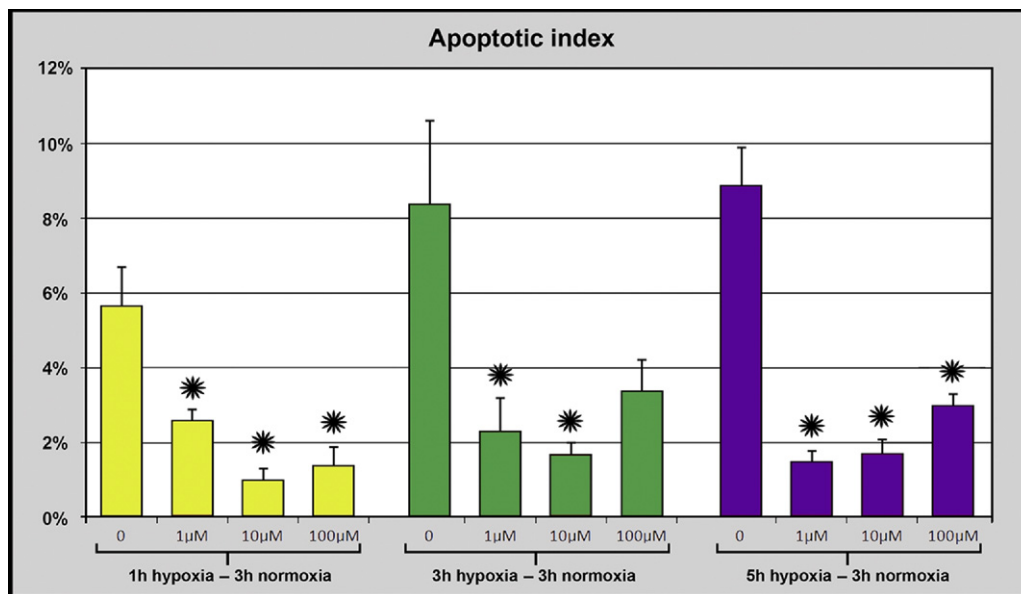
Fig 2. Myotube apoptotic index measured after 1, 3, and 5 hours of hypoxia, all followed by 3 hours of normoxia. The *star* denotes statistical significance compared with non-NaHS-treated control for each respective time. All cultures were repeated in triplicate. The *stars* indicate $P < .05$ relative to non-NaHS-treated cells at each time.
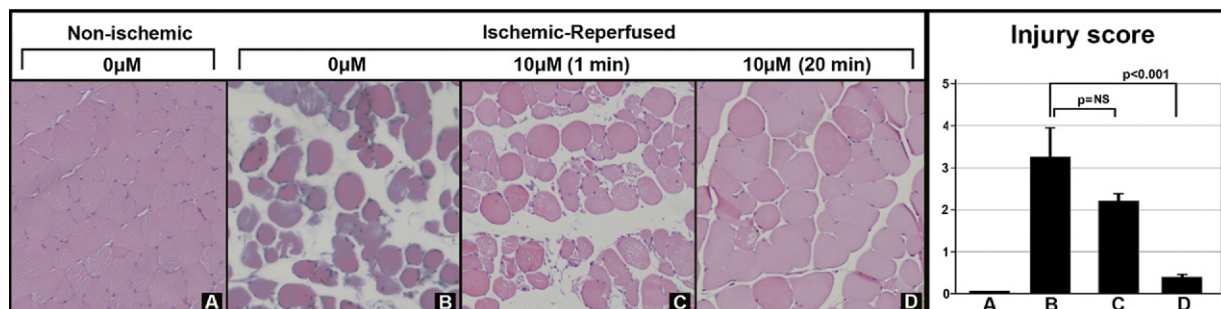

Fig 3. Photomicrographs show muscle after postischemic delivery of NaHS (n = 6 for each study group; original magnification, ×100). **A,** Injury score nonischemic control muscle, **(B)** ischemic-reperfused muscle that had not been treated with NaHS, **(C)** ischemic-reperfused tissue that had been treated with 10 μM NaHS 1 minute before reperfusion, and **(D)** ischemic-reperfused tissue that had been treated with 10 μM NaHS 20 minutes before reperfusion. Note the preservation of normal muscle architecture after treatment with NaHS 20 minutes before reperfusion but minimal preservation when delivered only 1 minute before reperfusion.

Our laboratory and others have previously shown the efficacy of pharmacologic preconditioning with HS in preventing the untoward effects of IRI in skeletal muscle.[23,27-30] HS is thought to be produced in most tissues throughout the body from L-cysteine, primarily by the enzymes cystathionine-β-synthase (CBS) and cystathionine-β-lyase (CSE). Multiple tissues types contain both of these enzymes, but CBS is thought to predominate in the central nervous system, and CSE predominates in the cardiovascular system.[31] HS is now known to have protean effects, including the regulation of inflammation and vascular permeability, vasomotor tone, and cellular metabolism among many others.

The mechanism through which HS confers protection against IRI remains largely unknown, but it is thought to be multifactorial (Fig 5). In addition to functioning as a direct scavenger of reactive oxygen species, HS appears to open mitochondrial K_{ATP} channels, which prevents mitochondrial hyperpolarization, calcium overload, and fatal opening of the mitochondrial permeability transition pore.[11,20] In addition, HS may activate prosurvival signaling cascades such as the reperfusion injury salvage kinases (RISK), which include PI3-kinase/Akt and extracellular signal-regulated kinase, among others.[32] Furthermore, the decrease in metabolism that results from HS may provide further protection from the ischemic interval by directly
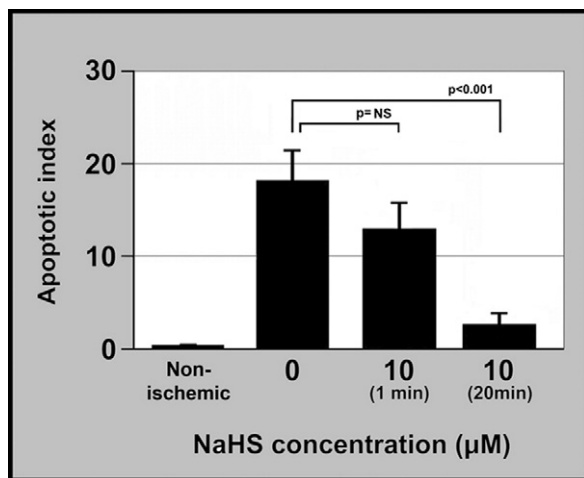
Fig 4. Muscle after postischemic delivery of NaHS (n = 6 for each study group). Apoptotic index for nonischemic control muscle, ischemic-reperfused muscle that had not been treated with NaHS, ischemic-reperfused tissue that had been treated with 10 μM NaHS 1 minute before reperfusion, and ischemic-reperfused tissue that had been treated with 10 μM NaHS 20 minutes before reperfusion. The *error bars* show the standard deviation.

Fig 5. Proposed multifactorial mechanism by which HS is cytoprotective against ischemia-reperfusion injury when delivered after the onset of ischemia. HS present in the cell can be exogenous or endogenous (produced by cystathionine-β-synthase [*CBS*] and cystathionine-β-lyase [*CSE*]) in nature and is thought to act as a free radical scavenger, an activator of the reperfusion injury salvage kinase pathway (*RISK*) pathway, and an opener of K_{ATP} channels, all of which promote cell survival via mitochondrial protection. *ERK,* Extracellular-regulated kinase; *MPTP,* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

decreasing the generation of substrates for reactive oxygen species upon reperfusion. This last mechanism, however, would not appear to be relevant to protection conferred by postconditioning.

In our previous studies, HS delivered 20 minutes before the onset of 3 hours of ischemia, followed by 3 hours of reperfusion, abolished evidence of IRI.[21,23] Our data also indicated that optimal protection was conferred when exogenously delivered HS increased the intravascular HS concentration to 10 μM. Although pretreatment of tissues with HS would certainly provide benefits in clinical scenarios of anticipated ischemia, as noted above, acute lower extremity ischemia in the clinical setting is usually impossible to anticipate. Therefore, based on the promising results from our studies evaluating the "preischemic" delivery of HS, we sought to determine whether HS could provide protection even when delivered after the onset of ischemia and therefore not reach the target tissue until after reperfusion.

As our data demonstrate, pharmacologic postconditioning with NaHS delivered after the onset of 1, 3, or 5 hours of hypoxia is capable of protecting cells against simulated IRI in vitro. Furthermore, when HS is delivered in vivo after 3 hours of ischemia but before reperfusion, similar protection was conferred to skeletal muscle. More important, the ischemic intervals used in these studies extended well beyond the established clinical limit of 2 hours of ischemia, after which point irreversible damage begins to occur in muscle tissue.[33] This is significant because in the clinical arena, all cases of unanticipated tissue ischemia and even most scenarios of anticipated tissue ischemia will last several hours. Thus, to make treatment
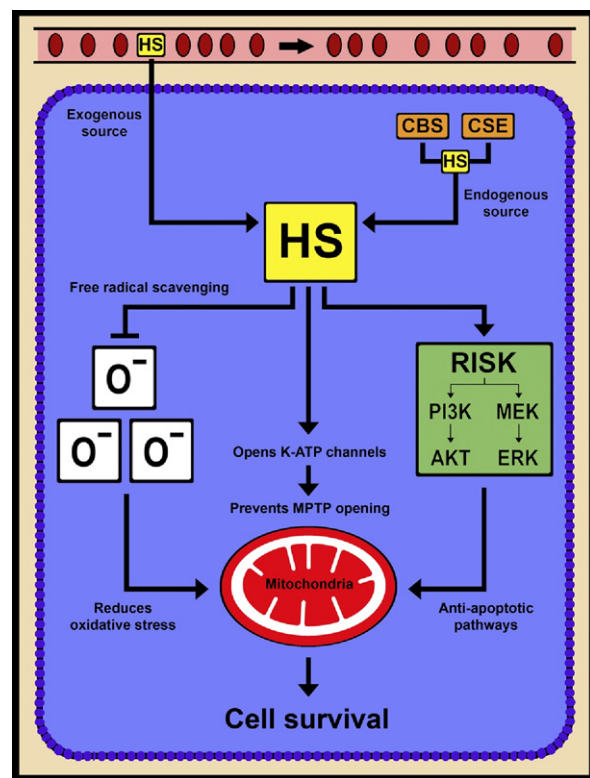
with HS a translatable therapy, efficacy must be demonstrated after several hours of ischemia. We are currently investigating the maximal period of ischemia that can be salvaged after ischemia and before reperfusion by treatment with NaHS.

Interestingly, the timing of HS delivery before reperfusion was crucial in our in vivo model. When delivered only 1 minute before reperfusion, no protective effect was seen; however, when given 20 minutes before reperfusion, a nearly complete protective effect was noted. The lack of protection seen with the later time point may be a result of an incomplete distribution of the HS, or more likely indicates that a finite interval of time is required to affect the relevant protective pathways (eg, activation/phosphorylation of cell survival and antioxidant pathways).[34]

The lack of protection noted when HS was given 1 minute before reperfusion in this study contrasts with the findings of Elrod et al,[27] who showed significant protection in cardiac tissue when HS was given at the time of reperfusion. Other authors have shown efficacy at 5 and 10 min-

utes before reperfusion.[22,35] Questions regarding the optimal time course of delivery, whether HS should be delivered as a bolus or as a continual infusion, and what is the optimal HS donor warrant further study.

CONCLUSIONS

This study is the first, to our knowledge, to concurrently evaluate both in vitro and in vivo the cytoprotective effects of HS on skeletal muscle when delivered after an ischemic event, and the results support our hypothesis that pharmacologic postconditioning with HS significantly limits IRI-induced damage. Although significant work remains to be done regarding translation to larger animal models and humans, we believe that these data provide an important foundation to suggest that treatment with HS prior to revascularization may prevent the morbidity (and possible mortality) that can result from unanticipated ischemia in skeletal muscle, and further work on this promising treatment modality is warranted.

AUTHOR CONTRIBUTIONS

Conception and design: PH, JS
Analysis and interpretation: PH, NJ, JR, AS, AW, DK, JS
Data collection: PH, NJ, JR, AS, DK
Writing the article: PH, NJ, JR, AS, DK, AR
Critical revision of the article: PH, AR, JS
Final approval of the article: JS
Statistical analysis: PH, AW
Obtained funding: PH, JS
Overall responsibility: JS

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