# ANTECEDENT HYDROGEN SULFIDE ELICITS AN ANTI-INFLAMMATORY PHENOTYPE IN POSTISCHEMIC MURINE SMALL INTESTINE

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Antecedent Hydrogen Sulfide Elicits an Anti-intlammatory
Phenotype in Murine Small Intestine

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And hereby certify that, in their opinion,
It is worthy of acceptance.

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LIST OF ABBREVIATIONS

ACE – angiotensin converting enzyme

Ang II – angiotensin II

$BK_{Ca}$ channel – large conductance calcium activated potassium channel

CGRP – calcitonin gene-related peptide

CO – carbon monoxide

eNOS – endothelial nitric oxide synthase

Glib - glibenclamide

5-HD – 5-hydroxydecanoate, selective mitochondrial ATP-sensitive potassium channel inhibitor

HMEC – human microvascular endothelial cell

HMR-1098 –selective plasmalemmal ATP-sensitive potassium channel inhibitor

HO – heme oxygenase

$H_2S$ – hydrogen sulfide

Ibx - iberiotoxin

I/R – ischemia/reperfusion

$K_{ATP}$ channel – ATP sensitive potassium channel

LA – leukocyte adhesion

LEI – leukocyte-endothelial interactions

LR – leukocyte rolling

MAPK – mitogen activated protein kinase

MCC-134 –selective plasmalemmal ATP-sensitive potassium channel opener

NO – nitric oxide

ROS – reactive oxygen species

ABSTRACT

Ischemia followed by reperfusion (I/R) is now well-recognized as one form of acute inflammation in which leukocytes play a key role. Recognition of the importance of the inflammatory process to the pathogenesis of I/R injury has led to an intensive research effort directed at identifying strategies to prevent leukocyte infiltration into post-ischemic tissues. Indeed, work conducted over the past 15 years has led to the development of the concept that oxidant-induced leukocyte/endothelial cell interactions are largely responsible for the microvascular dysfunction induced by reperfusion.

Preconditioning is a phenomenon through which antecedent exposure to a particular stimulus confers protection against a subsequent prolonged ischemic event. The development of a protected phenotype occurs in response to a diverse array of preconditioning stimuli, including short periods of ischemia, heat shock, ethanol, lipopolysacharide, calcitonin gene-related peptide, adenosine and bradykinin. Each of these preconditioning stimuli appears to promote the production of the gaseous monoxide, nitric oxide (NO), as an initial triggering event in the acquisition of tolerance to I/R. Recent work has shown that NO acts as an endogenous regulator of a second gaseous signaling molecule with vasorelaxant properties, hydrogen sulfide ($H_2S$). While it had been assumed that $H_2S$ acts solely as a toxic, environmental pollutant with minimal physiological and pathophysiological significance, it is now apparent that $H_2S$ is also synthesized endogenously in mammalian tissue. Furthermore, the vasorelaxation induced by NO is synergistically affected 13-fold by $H_2S$. Additionally, the production of $H_2S$ is up-regulated by NO.

These observations suggest that, like NO, $H_2S$ has the ability to fulfill a physiologic role in regulating cardiovascular function, distinct from its toxicologic effect. In light of these observations, we postulated that $H_2S$ inhibits inflammation after I/R injury, through four separate, yet not necessarily distinct, mechanisms. Although the temporal relationships of these mechanisms are important, this project has focused on the involvement of each mechanism, without specific elucidation of sequential order. The aims of this dissertation addressed the hypothesis that $H_2S$ elicits a preconditioning stimulus and protects against I/R injury through an eNOS-/p38 MAPK-/K channel-/HO-1 dependent mechanism.

## REVIEW OF THE LITERATURE

### Introduction

Ischemia, or inadequate supply of blood to a part of the body, is caused by partial or total occlusion of an artery. Early restoration of blood flow to ischemic tissues is an absolute prerequisite to halt the progression of cellular injury associated with decreased oxygen and nutrient delivery following ischemia. Although minimizing ischemic time is an important intervention for diminishing the extent of ischemic injury, reperfusion itself may lead to accelerated and additional tissue injury beyond that generated by ischemia alone (11, 56). It is now clear that reperfusion of ischemic tissues initiates a complex series of pathologic events that produce the same end result as prolonged hypoxia, i.e. cellular dysfunction and necrosis, collectively referred to as 'reperfusion injury'. (26, 30, 31, 67, 71). Recognition of the fact that reperfusion can initiate a cascade of deleterious processes that exacerbate the tissue injury induced by ischemia has resulted in an intensive research effort directed at defining the cellular and molecular events that underlie reperfusion injury.

One proposed mechanism of reperfusion injury involves the generation, accumulation and release of various reactive species along with simultaneous consumption of endogenous antioxidants. Reinfusion of previously ischemic tissue with hypoxic blood prevents reperfusion injury, while gradually increasing oxygen concentration in the reperfusate progresses injury, as with normoxic blood (73). These studies provide direct evidence that postischemic cell damage occurs by an oxygen-dependent mechanism. Indirect evidence supporting the role of ROS in I/R

has been the in vivo and in vitro cardioprotective effects of free radical scavengers and the beneficial effects of therapeutic agents supplementing or inducing antioxidants such as glutathione peroxidase (GSHPx) and superoxide dismutase (SOD). Increasing evidence based on genetic approaches, whereby overexpression or genetic deletion of genes participating in the antioxidant defense exhibits major influence on biochemical events, function, and outcome of I/R injury. Finally, electron spin resonance analyses of postischemic tissue samples show production of radical species. Indeed, ischemia and reperfusion is characterized by the accumulation of radical species, followed by characteristic changes in antioxidant defense systems (51, 92). As a result of oxidative stress, several cellular processes become dysregulated with damage occurring in every major cellular component, including membrane lipids, protein, carbohydrates, and DNA. The pathophysiological consequences of such uncontrolled injury are widespread tissue damage and associated inflammation, ultimately leading to cell death.

Objectives

The objectives of the studies outlined in this dissertation were: 1) to verify that antecedent hydrogen sulfide can produce a preconditioned phenotype in the endothelium of murine small intestine subsequently exposed to ischemia and reperfusion, 2) to determine whether NO, p38 MAPK, $K_{ATP}$ channels and $BK_{Ca}$ channels are the triggers of the preconditioned phenotype elicited by hydrogen sulfide, 3) to determine whether heme oxygenase-1 is a potential effector molecule required to produce the delayed protective effect of acute hydrogen sulfide

exposure, and 4) whether angiotensin II can mediate the deleterious effects of ischemia and reperfusion injury in the proposed scheme.

The hypotheses were as follows: 1) eNOS derived NO is required to elicit delayed $H_2S$ preconditioning in the endothelium, 2) p38 MAPK is required to elicit delayed $H_2S$ preconditioning in the endothelium, 3) $K_{ATP}$ channels are required to elicit delayed $H_2S$ preconditioning in the endothelium, 4) $BK_{Ca}$ channels are activated by H2S and are required to elicit delayed H2S preconditioning in the endothelium, and 5) HO-1 is an effector molecule upregulated secondary to H2S-PC that confers the anti-inflammatory phenotype, and 6) angiotensin II is responsible for the deleterious effects of ischemia and reperfusion injury.

## Mechanisms of Ischemia and Reperfusion Injury

Several key cellular changes result from the formation of reactive oxygen metabolites in post-ischemic tissues. Initially, the ROS are derived from NADPH oxidase (NOX) and xanthine oxidase (XO). I/R triggers a conversion of the normal xanthine dehydrogenase to the superoxide producing XO, thus providing a source of oxidants in postischemic tissue (42). Subsequently, during reperfusion, accumulating neutrophils in postischemic tissues produce large quantities of ROS. The superoxide anion itself is not a very potent radical, however, the conversion of superoxide to hydrogen peroxide by superoxide dismutase produces the cytotoxic species that interacts with low molecular weight metals, iron and copper, within the cell to produce highly reactive radicals. The formation of highly reactive free radicals ultimately promotes the release of pro-inflammatory stimuli and the expression of cell adhesion molecules on the leukocyte and endothelial cell surface. In addition, the ROS scavenge cellular NO, thereby removing the anti-adhesive effects of NO from the already compromised tissues and promoting further leukocyte-endothelial interactions (16). Once recruited to post-ischemic microvessels, activated neutrophils adhere to the surface of the endothelium and begin to migrate into the underlying tissues. Coincident with these redox changes, perivascular cells (e.g., macrophages, mast cells) become activated and release other inflammatory mediators (e.g., histamine, cytokines, $LTB_4$). As a consequence of these events, leukocytes begin to form adhesive interactions with postcapillary venular endothelium. More recent work indicates that platelets play an important role in the adhesion of leukocytes to the post-ischemic microvasculature, possibly by binding to

endothelial cells and providing a P-selectin rich platform onto which leukocytes can roll and adhere. The activated leukocytes emigrate into the tissues, inducing a microvascular barrier dysfunction via release of oxidants and hydrolytic enzymes. Thus, enhanced vascular protein leakage is one of the earliest signs of the microvascular dysfunction elicited by I/R (26, 30, 31, 67, 71).

The process of leukocyte adhesion and migration into post-ischemic tissues occurs in four distinct phases: tethering, rolling, arrest, and finally emigration into perivascular tissues (aka diapedesis) (58). In order for diapedesis to occur, the adhesive forces tethering leukocytes to the venular surface must exceed the shear forces that normally carry leukocytes away from areas of inflammation. Ischemic vessels exhibit much lower shear rates compared to normally perfused vessels, naturally enhancing the availability of leukocyte-endothelial interactions (21, 79). During reperfusion, shear rates increase back to normal rates, allowing only the force of cellular adhesion molecules (CAMs) to produce the tethering necessary to overcome shear stress.

The adhesion molecule families, along with the selectins, are the primary regulators of the coordinated sequence ultimately leading to leukocyte adhesion and migration. E-selectin and P-selectin are expressed on the surface of the endothelium and initiate the tethering that initiates rolling of the leukocytes along the vessel wall. During this rolling phase, leukocytes are exposed to and become activated by chemo-attractants and intracellular signals. Strengthening of the leukocyte-endothelial interactions occurs primarily through ICAM-1 and CD11/18-

mediated mechanisms. Finally, extravasation of activated leukocytes into the subendothelial space propagates the inflammatory process.

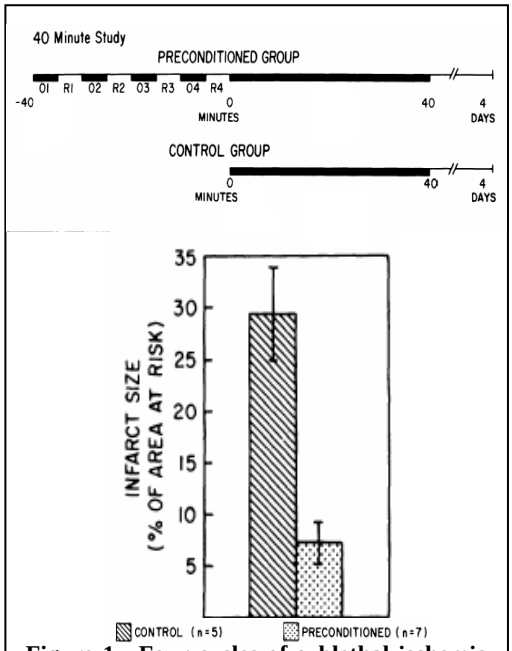


**Figure 1. Four cycles of sublethal ischemia preconditions against a subsequent ischemic event. (adapted from Murry et al. 1982)**

## Ischemic Preconditioning

Acute coronary occlusion is the leading cause of morbidity and mortality in the Western world and according the World Health Organization will be the major cause of death in the world as a whole by the year 2020 (60). Although the management of this epidemic is centered on the development of effective primary prevention, the impact of these strategies is limited and necessitates urgent need for secondary forms of prevention. Aside from the therapeutic potential in coronary syndromes, other clinically relevant ischemic events (e.g., thromboembolic stroke, mesenteric ischemia, cardiopulmonary bypass, angioplasty and organ transplantation) would benefit from the same line of therapy.

The single greatest advance in the field of I/R came in 1986 when Murry et al. showed that four cycles of 5 min ischemia with intermittent reperfusion would limit infarct size by 75% (figure 1), an unprecedented amount of protection (61). This phenomenon, termed ischemic preconditioning (IPC), has been so profound that it has been recognized as ‘the strongest form of in vivo protection agains myocardial ischemic injury other than early reperfusion (40). Not surprisingly, IPC has been the subject of enormous research over the last 20 years. Ischemic preconditioning is

associated with two forms of protection: a classical form lasting ~2h after the preconditioning ischemia followed 24 h later by a second window of protection lasting ~72 h (figure 2). Both windows of preconditioning trigger protection by similar mechanisms: the release of autocoids that confer protection by occupying cell surface receptors. Receptor occupancy then triggers a cascade of signaling events that converge upon one of more effects during the lethal ischemic event to mediate protection. The prolonged protection conferred by the second window of preconditioning suggests this window may be clinically useful to produce a sustained resistance to ischemia. This proposal will focus on the potential pharmacologic exploitation of this late phase of preconditioning.

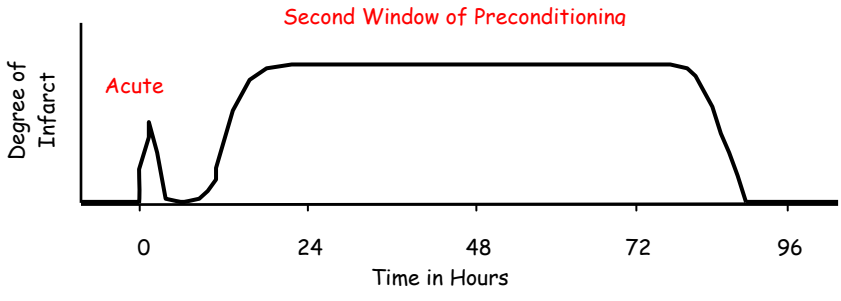


**Figure 2. Proposed temporal relationship of $H_2S$ preconditioning. The second window of preconditioning represents the 'late phase'.**

In order to characterize the signaling cascades responsible for the preconditioned phenotype, it is necessary to review terminology that has been established to specify temporal and mechanistic relationships of the preconditioning stimulus. 1) *Triggers* or *initiators* of the preconditioning stimulus include the same molecular species that are seen elevated following the first sublethal ischemic challenge and are responsible for initiating the adaptation; 2) *mediators* are the molecular species that are expressed 24-72 h later and are responsible for

conferring protection during the second ischemic challenge; and 3) *effectors* that are the signaling pathways that are activated by the triggers and culminate in the expression of the mediators (6).

Although specific details regarding the intracellular pathways leading to the late phase of preconditioning have not been elucidated, the basic schema of this phenomenon is already known. Initiators of preconditioning (e.g. adenosine, bradykinin, ROS, NO and opioids) commonly bind to G-protein coupled receptors, causing phosphorylation cascades, activation of kinases, activation of transcription factors and finally regulation of gene products that ultimately lead to the remarkable adaptation and tolerance to ischemic stress (figure 3) (6). As our understanding of the diverse signaling cascades following preconditioning is unraveled, the possibility to manipulate these mechanisms with novel pharmacologic therapeutics may become more of a reality.

Ischemia/reperfusion injury occurs at the level of the microvasculature, however, many models can potentially fail to produce definite conclusions in regards to microvascular changes after ischemic preconditioning or I/R. Isolated perfused organs serve to deliver biochemical markers of

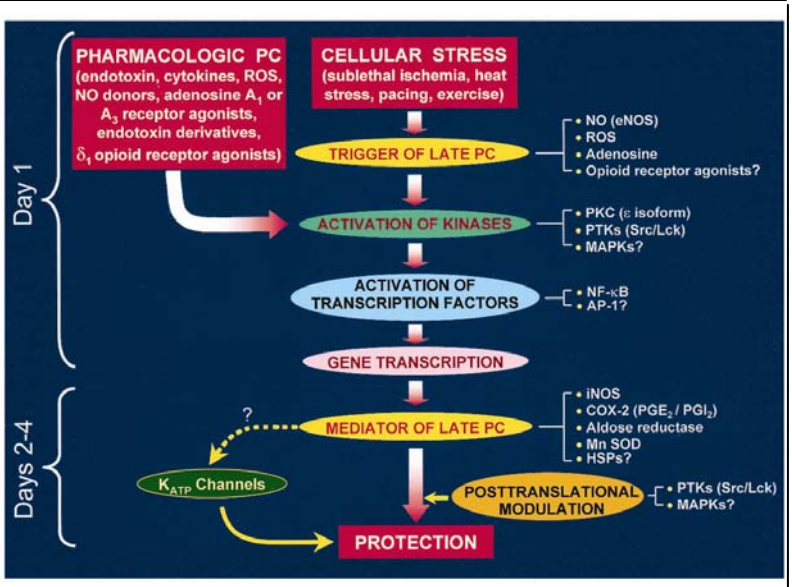


**Figure 3. Intracellular signaling pathways leading to the preconditioned phenotype (Bolli, Circ Res 2000)**

injury, as well as indices of tissue necrosis, however they serve to define the final result of protective interventions, without elucidating the character of the microvascular process as well. For example, the Langendorf perfused heart model of I/R does not simulate ischemia, since the formed elements of blood are not actually present. Furthermore, although tissue culture approaches advance science with the ability to isolate cell-type specific responses and powerfully investigate changes in proteins, gene transcription and cellular messenger molecules, they lack the physiological milieu and relevance of whole organ preparations. The microvascular models utilizing intravital microscopy or isolated perfused vessels present a balance of specificity and physiological relevance necessary in the study of preconditioning phenomena. The most ideal investigation of ischemia/reperfusion injury and the effects of pharmacologic manipulations to precondition the vessels would include a combination of all of these models.

*Initiators of Ischemic Preconditioning*

Clinically relevant application of the preconditioning phenomena is best applied by elucidating the mechanisms and cellular triggers of the preconditioning cascade. These triggers are usually mildly noxious stimuli that upregulate the endogenous protective phenotype, that if in place during a subsequent, more deleterious ischemic event, could protect the vessels and tissue from injury.

It is well known that bioactive substances accumulate in post-ischemic tissues and that small non-poisonous levels of these same bioactive substances could

trigger complex cellular responses and induce an adaptive transformation into a protected phenotype.

Reactive Nitrogen and Oxygen Metabolites

ROS and RNOS are emerging as critical signaling molecules (20). The ROS encompass a wide range of molecules. Free radicals are chemical species containing one or more unpaired electrons (32). Examples include the hydrogen atom, most transition metal ions, nitric oxide, and oxygen. The 2 unpaired electrons of oxygen react to form partially reduced highly reactive species that are classified as ROS, including superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite. Various enzyme systems produce ROS, including the mitochondrial electron transport chain, cytochrome P450, lipoxygenase, cyclooxygenase, the NADPH oxidase complex, xanthine oxidase, and peroxisomes. Mitochondrial oxygen metabolism is the dominant source of superoxide that results from incomplete coupling of electrons and hydrogen atoms with oxygen in the electron transport chain (20, 47, 49, 98).

Under normoxic conditions, ROS are maintained within a narrow boundary by scanvenging systems, as would be expected where fluxes of ROS would be involved in cell signaling. Redox balance, the ratio between oxidizing and reducing species within the cell, plays a significant role in the regulation of signaling pathways, including kinase and phosphatase activity and gene expression (90). Redox balance is achieved by various enzyme systems that neutralize toxic oxidants, such as ROS/RNOS. Superoxide dismutases catalyze the conversion of superoxide to hydrogen peroxide, which can then be converted to water by catalase or glutathione

(GSH) peroxidase coupled with glutathione reductase. Other endogenous free radical scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which uses reduced glutathione (GSH) as a substrate (52). GSH plays a critical role in maintaining redox homeostasis and the GSH to oxidized glutathione ratio provides an estimate of cellular redox buffering capacity.

Interestingly, reperfusion after prolonged ischemia is associated with a burst of ROS and RNOS production at levels which produce apoptosis and necrosis in unpreconditioned tissues (13). IPC prevents this harmful oxidant burst at reperfusion (94). It is now well established that reactive oxygen (ROS) and reactive nitrogen oxide species (RNOS) act as triggers in both early and late phase preconditioning (13). Indeed, exposing cells to low levels of hydrogen peroxide as a PC stimulus abrogates leukocyte adhesion, reduces IL-6 and IL-8 secretion, and decreases adhesion molecule expression in vitro (101).

Angiotensin

The elucidation of the molecular machinery responsible for the protection afforded by ischemic preconditioning has provided several rational targets for pharmacological interventions to treat ischemia/reperfusion injury syndromes. Angiotensin II (Ang II) is the main active peptide of the renin-angiotensin system (RAS) that is known to exert a multitude of actions in many organs, including the heart, blood vessels, and the kidney (70). Although Ang II is necessary for physiological regulation of blood pressure and maintenance of fluid and electrolyte homeostasis, exogenous Ang II can be deleterious, inducing myocardial infarction and tubular necrosis (22). Among these deleterious effects, Ang II also promotes

hypertrophy and fibrosis (74). Interestingly, cardiac and vascular tissue possesses all of the components of the RAS, and Ang II is produced locally within the myocardium and blood vessels (4). Although inhibition of angiotensin converting enzyme and the Ang II type I receptor (AT1) has been proven beneficial in several cardiovascular pathologic conditions in humans, exogenous Ang II administration prior to sustained ischemia has also been shown to induce PC cardioprotection (17, 80).

In regards to PC, it is essential to emphasize the established role of ROS in Ang II intracellular signaling cascades (28). The precise molecular links that generate ROS via the NADPH oxidase complex following Ang II stimulation is well studied but remains unspecified (33). Interestingly, Ang II activates specific mitogen activated protein kinases (MAPKs) and antiapoptotic kinases in a ROS-dependent fashion (15). Ang II preconditioning is triggered by redox cycling of ROS that are generated by NADPH oxidase-dependent, by the increased activity of p22phox and gp91phox, and NADPH oxidase-independent pathways (27). Additionally, phosphorylation of p47phox occurs secondary to Ang II with concomitant induction of ROS formation via NADPH oxidase. NADPH oxidases are considered to be the major sources of ROS in vascular smooth muscle cells responsible for redox signaling (65). It is because of these associations of AngII-mediated increase in ROS and MAPK activity that Ang II mediated effects in the field of PC is imperative.

Large Conductance Potassium Channels

Large conductance, calcium-activated potassium ($BK_{Ca}$) channels are critical triggers for the development of the protected phenotype in response to

preconditioning stimuli (84, 89). These channels are expressed by parenchymal cells in a variety of organs, but also in vascular tissues, including endothelial and vascular smooth muscle cells. $BK_{Ca}$ channels can be activated by elevations in intracellular calcium and by membrane depolarization and demonstrate single channel conductances of ~250 pS. $BK_{Ca}$ channels are comprised of a pore-forming $\alpha$-subunit and a modulatory $\beta$-subunit that is sensitive to channel blocking agents (7). In the heart, BKCa channels are thought to be present only in mitochondria, whereas endothelial cells express these channels on the plasma membrane (25, 35).

Recent work demonstrates that endothelial $BK_{Ca}$ (also termed Slo or MaxiK) channels play a role in regulating synthesis of NO (36, 62, 83, 99). Additionally, preconditioning with the selective $BK_{Ca}$ opener NS1619 induces both early and late phase preconditioning in the myocardium, effects that were not blocked by $K_{ATP}$ channel antagonists but were mitigated by the selective $BK_{Ca}$ blockers, iberiotoxin or paxilline (81, 96, 100). Moreover, the cardioprotective effects of estradiol appear to involve $BK_{Ca}$ channel activation in rat ventricular myocytes exposed to simulated ischemia (59, 63). Importantly, endothelial cells do not express voltage-gated calcium channels, therefore, changes of intracellular calcium concentrations are due mainly to calcium release from internal stores or through transmembrane calcium influx, that is dependent of membrane hyperpolarization (62). Potassium channels have been shown to regulate endothelial membrane potential and thereby influence intracellular calcium levels (62). Thus, the protective effect of the BKCa channels has been proposed to occur by enhancing mitochondrial K+ uptake and in turn

reducing the mitochondrial Ca2+ overload that can occur secondary to ischemia (96).

*Downstream Signaling Elements*

ATP-sensitive Potassium Channels

Structurally, $K_{ATP}$ channels are composed of two distinct proteins, an inwardly rectifying potassium channel ($K_{ir}$) pore subunit and the sulfonylurea receptor (SUR), that may have a regulatory role as well as a function in modulating the sensitivity of the channel to ATP, other nucleotides, and pharmacological agonists or antagonists (29). It is currently known that the cardiac sarcolemmal $K_{ATP}$ channel is composed of an octomeric complex of two types of subunits, the Kir6.2 and the SUR2A subunit. There is also evidence to suggest that there are two $K_{ATP}$ channels in the cell, a sarcolemmal channel (sarc$K_{ATP}$ channel) in which the structure has been clearly delineated and a putative channel in the inner mitochondrial membrane (mito$K_{ATP}$ channel). The mito$K_{ATP}$ channel has been characterized pharmacologically in cells and in isolated lipid bilayers; however, it has not been cloned and its molecular structure remains unknown. Whereas there are multiple potential sources of oxidants, it has been suggested that uncoupling oxidative phosphorylation in mitochondria can induce general oxidative stress which in turn may trigger opening of mitochondrial $K_{ATP}$ channels ($mK_{ATP}$) (53, 97). ATP inhibits the $mK_{ATP}$ channel, therefore the reduction of ATP stores during ischemia may be responsible for opening of the $mK_{ATP}$ channel.

Considerable controversy exists defining the role of $K_{ATP}$ channel subtypes in preconditioning and specifying the subtype responsible for the preconditioning effect. Recent data suggest that both the sarc$K_{ATP}$ and mito$K_{ATP}$ channels play complimentary roles in the protection afforded by IPC. On the basis of recent evidence, activation of the mito$K_{ATP}$ channel appears to limit cell death, whereas opening of the sarc$K_{ATP}$ channel appears to limit stunning. Regardless, direct activation of either the sarc$K_{ATP}$ or mito$K_{ATP}$ channels provide significant cardioprotection.

p38 Mitogen Activated Protein Kinases

The MAPKs have been shown to play a crucial role in ischemic preconditioning. In addition, several MAPKs including Erk (1/2), JNK and p38 MAPK are main targets for ROS signaling (15, 45, 78). Unlike Erk (1/2), which is activated by growth signal via Ras-dependent signaling transduction pathways, activation of JNKs and p38MAPK are potentiated by diverse stresses and proinflammatory cytokines (87). Indeed, downstream signaling elements that are activated by ROS as a result of IPC include free radical-MAPK signaling (14).

A number of recent studies implicate the activation of p38 MAPK in development of preconditioned states (3, 5, 38). At least 6 different isoforms of p38 MAPK have been described. This MAPK is activated by dual phosphorylation on a Thr-Gly-Tyr motif in response to a number of stimuli implicated as triggers for preconditioning, including antecedent exposure to short bouts of ischemia or NO donors (12, 41, 45, 50, 64).

Calcitonin Guanine-Related Peptide

CGRP is a proadhesive peptide well known for its role in neurogenic inflammation (2, 82). CGRP is a major transmitter in capsaicin-sensitive sensory nerves and has two isoforms named $CGRP_{\alpha}$ and $CGRP_{\beta}$. CGRP interacts with its receptor to produce several physiopharmacological effects, such as positive inotropic actions, vasorelaxation, and protective effects of myocytes and endothelial cells (44). There exist at least two classes of CGRP receptors, $CGRP_1$ and $CGRP_2$ (8, 43). The receptors belong to the rhodopsin-like superfamily of G-protein-coupled receptors (68). Cardiovascular effects of CGRP are mediated by the $CGRP_1$ receptor, which can be blocked by the CGRP receptor antagonist, CGRP-(8–37).

Recently, it has been shown that CGRP plays an important role in mediation of ischemic preconditioning (44, 46, 72). In the isolated perfused rat heart, the CGRP concentration in the coronary effluent was elevated during the preconditioning period. Ischemic preconditioning significantly improved recovery of cardiac function during reperfusion after 30 min of global ischemia, and this cardioprotection by ischemic preconditioning was abolished by CGRP-(8–37), a selective $CGRP_1$ receptor antagonist. Furthermore, delayed cardioprotection or gastroprotection by intestinal or gastric preconditioning is mediated by CGRP (66, 69, 85, 86).

*Effector Molecules: HO-1*

Heme oxygenase (HO) is a ubiquitously expressed protein that catalyzes the oxidative degradation of protoheme IX into equimolar quantities of biliverdin, divalent

iron, and carbon monoxide (CO). Biliverdin is further metabolized to bilirubin, a powerful endogenous antioxidant, by the action of biliverdin reductase. Three isoforms of the enzyme, HO-1, HO-2,and HO-3, have been described (48). HO-3 appears to exhibit lower activity and is less well characterized than HO-1 and HO-2, and may be a splice variant of HO-2. HO-2 is a constitutively expressed and non-inducible gene product. On the other hand, HO-1 is an inducible enzyme which is regarded as a heat shock protein (HSP32) in animal models.

HO-1 induction in models of oxidative stress has been shown to protect against noxious stimuli, including ultraviolet radiation, hyperoxia, LPS, and heme-induced injury, in vitro and in vivo. There is an increased susceptibility of HO-1 knockout mice to oxidative stress and a similar pattern in the case of human HO-1 deficiency, further supporting the role of HO-1 in the host defense against oxidant injury. Several investigators have used ischemia and reperfusion injury to confirm that increased expression of HO-1, or its reaction product carbon monoxide, correlates with improved survival and organ function in the brain, kidney, liver and lungs (75, 76, 91).

Despite much work to show the protective effect of HO-1, much less work has been done to determine the mechanism of HO-1 induced protection. Previous studies have implicated cis-regulatory elements in the HO-1 gene. Upstream signaling targets also include MAPKs (103).

## Hydrogen Sulfide as an endogenous gaseous signaling molecule

Although once only regarded as a toxic gas, the newly identified endogenous gaseous signaling molecule, $H_2S$, affects the structures and functions of the human body at molecular, cellular, tissue and system levels (1, 39, 54, 95). $H_2S$ is produced in mammalian tissues primarily through the activity of two pyridoxal-5'-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). Specifically, the generation of $H_2S$ from vascular tissues is due mainly to the specific irreversible catalytic activity of CSE (57). The endogenous substrates yielding production of $H_2S$ include sulfur containing amino acids L-cysteine and methionine. $H_2S$ can be generated by the spontaneous dissociation of the $H_2S$ donor, sodium hydrosulfide (NaHS), in acqueous solution according to the equations:

$$NaHS \rightarrow Na^+ \text{ and } HS^-$$

$$2HS^- \leftrightarrow H_2S + S^{2-}$$

$$HS^- + H^+ \leftrightarrow H_2S$$

### *Role of $H_2S$ in Cardioprotection*

The pathophysiological roles of $H_2S$ in the myocardium and coronary vasculature have received little attention. Recent reports indicate that $H_2S$ is spontaneously generated in the myocardium from CSE at concentrations in the micromolar range and that both endogenous and exogenous $H_2S$ produce marked cardiac effects (37).

In rat heart, exogenous $H_2S$ has been shown to produce concentration and time-dependent decreases in left ventricular $dP/dt_{max}$ (24). This negative inotropic effect was abolished by glibenclamide treatment. Furthermore, downregulation of CSE gene expression, enzyme activity and $H_2S$ production is shown in an in vivo rat model of myocardial injury. This effect was induced by subacute isoprenaline in vitro, and exogenous $H_2S$ reduced markers of lipid peroxidation in myocardial homogenates exposed to ROS (23).

The initial evidence demonstrating the endogenous role $H_2S$ exerts on the vascular system showed that a low concentration of $H_2S$ enhances the smooth muscle relaxation effect of NO 13-fold (34). In the same study, other thiols did not relax smooth muscle by themselves and did not have a synergistic relaxation effect with nitric oxide, suggesting the response is specific to $H_2S$. Since these initial studies, other studies have confirmed the vasoregulatory effects of $H_2S$ (18, 77, 88, 93). Acutely, NO increases CSE activity, while chronically, NO up-regulates the expression of CSE (95, 104). Nevertheless, it is apparent that the crosstalk between NO and $H_2S$ necessitates further characterization.

Apparent to the functional significance of $H_2S$, CBS deficiency leads to hyper-homocysteinemia, hypertension and endothelial dysfunction (9, 10, 55). In rodents, genetic deficiency of CBS/CSE or chronic treatment with DL-propargylglycine, an irreversible and selective inhibitor of CSE, results in reduced NO bioactivity. Either condition leads to severe endothelial dysfunction with impaired aortic relaxation to acetylcholine and a paradoxical vasoconstriction of mesenteric microvessels in response to bradykinin (19).

*Role of $H_2S$ in Non-Cardiac Tissues*

$H_2S$, endogenous and exogenous, has been implicated in non-cardiac tissues to affect, specifically the colon, liver, and lung. Comparable to the case of NO, $H_2S$ has been implicated to prevent tissue damage and inflammation and as an agent that causes tissue damage and inflammation. In the context of the gastrointestinal system, $H_2S$ maintains mucosal integrity, regulates blood flow, and modulates inflammation (102). Deficiencies of $H_2S$ may even contribute to several gastrointestinal and liver disorders. Non-steroidal anti-inflammatory drugs, including aspirin, can suppress the expression of one of the key enzymes required for $H_2S$ synthesis, and suggests that this could be another mechanism contributing to the gastrointestinal damage produced by this widely used class of drugs.

The roles for $H_2S$ in inflammation and immunity have been identified, but not yet clearly defined. This is apparent in much data that points to both inflammatory and anti-inflammatory effects of $H_2S$.

## Summary of the Literature

Although several pharmacologic agents have been shown to recapitulate the protective effects of IPC, utilization of this phenomenon in the clinical arena has been limited by incomplete understanding of the complex molecular pathways that follow IPC. These important observations may potentially be utilized to modulate activities of endogenous regulators of ischemia for therapeutic purposes.

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# HYDROGEN SULFIDE TRIGGERS LATE PHASE PRECONDITIONING IN POSTISCHEMIC SMALL INTESTINE BY AN eNOS- AND p38 MAPK-DEPENDENT MECHANISM

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*Running head:* Hydrogen sulfide preconditioning

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## ABSTRACT

Hydrogen sulfide ($H_2S$) is one of three endogenous gases, along with carbon monoxide (CO) and nitric oxide (NO), that exerts a variety of important vascular actions in vivo. Although it has been demonstrated that CO or NO can trigger the development of a preconditioned phenotype in postischemic tissues, it is unclear whether $H_2S$ may also induce protection in organs subsequently exposed to ischemia/reperfusion (I/R). Thus, we postulated that preconditioning with an exogenous $H_2S$ donor (NaHS-PC) would inhibit leukocyte rolling (LR) and adhesion (LA) induced by subsequent exposure to I/R. We used intravital microscopic techniques to demonstrate that NaHS-PC 24 hrs, but not 1 hr, prior to I/R causes postcapillary venules to shift to an anti-inflammatory phenotype in wild-type (WT) mice such that these vessels failed to support LR and LA during reperfusion. The protective effect of NaHS-PC on LR was largely abolished by coincident pharmacologic inhibition of NO synthase in WT animals and was absent in eNOS-deficient mice. A similar pattern of response was noted in WT mice treated concomitantly with NaHS plus p38 mitogen activated protein kinase (MAPK) inhibitors (SB203580 or SK86002). While the reduction in LA induced by antecedent NaHS was attenuated by pharmacologic inhibition of NOS or p38 MAPK in WT mice, the anti-adhesive effect of NaHS was still evident in eNOS-/- mice. Thus, NaHS-PC prevents LR and LA by triggering the activation of an eNOS- and p38 MAPK-dependent mechanism. However, the role of eNOS in the anti-adhesive effect of NaHS-PC was less prominent than its effect to reduce LR.



**Keywords:** ischemia, reperfusion, NaHS, eNOS-deficient mice, leukocyte rolling and leukocyte adhesion.

## INTRODUCTION

Although the noxious effects of hydrogen sulfide ($H_2S$) have long been recognized, it is now clear that this gaseous environmental toxicant is produced endogenously by the vasculature and many tissues, is present in micromolar concentrations in blood and brain, and modulates tissue function (51). Endogenous $H_2S$ is formed locally by two pyridoxal-5'-phosphate-dependent enzymes, cystathionine $\beta$-synthase (CBS) and cystathionine $\gamma$-lyase (CSE, also referred to as cystathionase) that exhibit a differential tissue distribution. CBS is highly expressed in the central nervous system where $H_2S$ has been shown to enhance NMDA receptor-mediated currents and inhibit CRP release (1, 27). On the other hand, CSE is expressed in the vascular wall of the aorta, tail artery, pulmonary artery, mesenteric artery and portal vein, while CBS is not detectable in these vessels (21, 62). Each of these enzymes utilize L-cysteine as a substrate to produce $H_2S$, which exists in physiologic solutions as $H_2S$ and $HS^-$ in a 30%:70% ratio. Because the molecular targets for these sulfide species are not well-described, we use $H_2S$ to refer to $H_2S$ and $HS^-$. A third $H_2S$-producing enzyme, 3-mercaptosulfurtransferase, has been demonstrated in the heart (25).

As an environmental pollutant, high concentrations (>250 $\mu$M) of $H_2S$ are toxic by virtue of its ability to complex with Fe3+ of mitochondrial cytochrome oxidase (thereby impeding cellular oxidative metabolism) and to inhibit carbonic anhydrase and tyrosine aminotransferase (51). Endogenous production of $H_2S$ results in plasma levels of 10-100 $\mu$M, much lower concentrations that allow this sulfide to subserve signaling functions that modulate vascular tone, baseline leukocyte adhesion, and neuronal activity (14, 51, 58, 59). In addition, exogenous administration of sodium hydrosulfide

(NaHS), a $H_2S$ donor, has been shown to relax vascular smooth muscle, attenuate the increase in leukocyte-endothelial cell adhesive interactions induced by proinflammatory mediators, and exert infarct-sparing effects in models of myocardial ischemia/reperfusion (I/R) (49, 51, 63). In this regard, $H_2S$ modulates cardiovascular function in a manner similar to the other two endogenously produced gaseous signaling molecules, nitric oxide (NO) and carbon monoxide (CO).

Preconditioning refers to a phenomenon whereby antecedent exposure to a particular stimulus confers protection against the deleterious effects induced by subsequent exposure to prolonged I/R. Preconditioning stimuli induce two phases of protection in I/R. Acute or early phase preconditioning develops rapidly (within minutes), involves activation of pre-existing effector molecules, and is short-lived, conferring protection for 2-6 hrs before disappearing. Delayed acquisition of tolerance to ischemia (late phase preconditioning) arises 12-24 hrs after the initial preconditioning stimulus is applied, is longer-lived (24 hrs or longer), and requires the expression of new gene products to mediate cardioprotection. The development of a protected phenotype occurs in response to a diverse array of preconditioning stimuli, including short periods of ischemia, heat shock, ethanol ingestion at low to moderate levels, lipopolysacharide, calcitonin gene-related peptide, $K_{ATP}$ channel agonists, adenosine and adenosine receptor agonists, opioids, and bradykinin (24). Many of these preconditioning stimuli appear to promote the production of the gaseous monoxide, nitric oxide (NO), phosphorylation and activation of p38 MAPK, and activation of plasmalemmal ATP-sensitive potassium ($K_{ATP}$) channels as initial triggering events in the acquisition of tolerance to I/R (11, 47), while formation of the diatomic gas, carbon monoxide (CO)

and activation of mitochondrial $K_{ATP}$ channels appear to serve as important effectors of protection during I/R (5, 34, 41, 52). In addition, preconditioning with NO donors or CO releasing molecules, p38 MAPK activators, and $K_{ATP}$ channel agonists prevents postischemic leukocyte infiltration and exerts infarct-sparing effects in postischemic tissues (55).

Several effects of $H_2S$ suggest a potential role for this gaseous signaling molecule as a preconditioning stimulus. $H_2S$ produces vasorelaxation by activation of $K_{ATP}$ channels and can enhance the vasorelaxant effects of NO (21). Conversely, the production of $H_2S$ can be up-regulated by NO (60). Both NO and $K_{ATP}$ channel activation produce preconditioned states. $H_2S$ also exhibits anti-adhesive effects, preventing leukocyte rolling and adhesion to mesenteric venules induced by coincident exposure to inflammatory mediators (14, 59). Based on these observations, we hypothesized that $H_2S$ exposure, using the $H_2S$ donor NaHS, either during reperfusion or as a preconditioning stimulus 1 (acute phase) or 24 hrs (late phase) prior to I/R, would prevent postischemic leukocyte rolling and adhesion. Since the results of these initial experiments indicated that $H_2S$ failed to prevent postischemic leukocyte/endothelial adhesive interactions when administered 1 hr prior to I/R (acute phase preconditioning) or during reperfusion but was remarkably effective at preventing these responses when administered as a preconditioning stimulus 24 hrs prior to I/R, we focused the remainder of our work on the mechanisms involved in triggering entrance into this anti-inflammatory state. We hypothesized that late phase NaHS-PC, 24 hrs prior to I/R would induce the development of an anti-inflammatory phenotype by an eNOS- and p38 MAPK-dependent mechanism.

## MATERIALS AND METHODS

*Animals:* Wild-type (WT) male C57BL/6J mice or eNOS -/- mice (6-7 weeks of age) were obtained from the Jackson Laboratories (Bar Harbor, ME). All mice were maintained on standard mouse chow and used at 8-12 weeks of age. The experimental procedures described herein were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri – Columbia Institutional Animal Care and Use Committee.

*Surgical Procedures and Induction of I/R:* The mice were anesthetized initially with a mixture of ketamine (150 mg/kg body wt, i.p.) and xylazine (7.5 mg/kg body wt, i.p.). The right carotid artery was cannulated and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery catheter. Systemic blood pressure was recorded continuously with a personal computer (Power Macintosh 8600; Apple) equipped with an analog-to-digital converter (MP 100; Biopac Systems). Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE, Molecular Probes, Eugene, OR, USA) was dissolved in DMSO at a stock concentration of 5 mg/ml, divided into 25 µl aliquots, and stored in light-tight containers at -20 °C until further dilution immediately prior to intravenous injection. The left jugular vein was cannulated for administration of CFDA-SE. A midline abdominal incision was then performed andthe superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (sham) or 45 min. After the ischemic period, the clip was gently removed and leukocytes were labeled with CFDA-SE by intravenous administration of the fluorochrome solution (250 µg/ml saline) at 20 µl/min for 5 min. During preparation, storage, and administration of CFDA-SE, care was taken to minimize light exposure.

Leukocyte/endothelial cell adhesive interactions were quantified over 30-40 and 60-70 min of reperfusion, as described below.

*Intravital Fluorescence Microscopy:* The mice were positioned on a 20 × 30-cm Plexiglas board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 × 3-cm hole centered in the Plexiglas. The exposed small intestine was superfused with warmed (37 °C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min using a peristaltic pump (Model M312; Gilson). The exteriorized region of the small bowel was covered with BBS-soaked gauze to minimize tissue dehydration, temperature changes, and the influence of respiratory movements. The superfusate was maintained at 37 ± 0.5 °C by pumping the solution through a heat exchanger warmed by a constant-temperature circulator (Model 1130; VWR). Body temperature of the mice was maintained between 36.5 and 37.5 °C by use of a thermostatically controlled heat lamp. The board was mounted on the stage of an inverted microscope (Diaphot TMD-EF; Nikon) and the intestinal microcirculation was observed through a 20× objective lens. Intravital fluorescence images of the microcirculation (excitation wavelength, 420-490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics) and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD; Sony) and recorded on digital video using a digital video recorder (DMR-E50; Panasonic) for off-line quantification of measured variables during playback

of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function on the monitor.

The intravital microscopic measurements described below were obtained durng minutes 30-40 and 60-70 of reperfusion or at equivalent time points in the sham control groups. The intestinal segment was scanned from the oral to aboral section and 10 single, unbranched venules (20-50 μm diameter, 100 μm length) were observed, each for 30 sec. Leukocyte-endothelial cell interactions (the numbers of rolling and firmly adherent leukocytes) were quantified in each of the 10 venules, followed by calculation of the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move or detach from the venular wall for a period equal to or greater than 30 sec. Rolling cells are defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than centerline velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adherent leukocytes were normalized by expressing each as the number of cells per $mm^2$ vessel area.

*Human Microvascular Endothelial Cells – (HMEC-1) Culture*: HMEC-1, a cell line derived from dermal human microvascular endothelial cells, were cultured as described previously (2) and maintained at 37 °C with 5% $CO_2$. The cells were seeded and grown in MCDB-131 (Sigma Aldrich, St. Louis, MO) medium containing L-glutamine and sodium bicarbonate (11.6g/L), glucose and 10% (v/v) fetal bovine serum (Invitrogen). The cells were initially seeded at a density of 5,000 cells/$cm^2$ and subcultured when they were 70-80% confluent (~10 days), then detached using 0.25% trypsin/0.5 mM EDTA in phosphate-buffered saline 5% $CO_2$. Cells are viable for at least 90 passages.

Protein expression was analyzed with Western blotting (9). Briefly, small 15% SDS gels were used. After transfer of proteins and molecular weight markers to nitrocellulose, the membrane was blocked with 5% milk in phosphate buffered saline with 0.5 % tween (PBS-T). The membrane was then incubated with appropriate primary antibodies. Horseradish peroxidase-coupled secondary antibodies labeled with were used to detect the primary antibodies. Pierce Supersignal West Pico Chemiluminescent Substrate was used to visualize protein expression bands on autoradiography film.

*Experimental Protocols:* The general design of the experimental protocols for each group in the study is described below and in Figure 1.

***Group 1: Sham.*** As a time control for the effects of experimental duration, the mesentery of each mouse in this group (n=6) was superfused with bicarbonate-buffered saline. The superior mesenteric artery was exposed but not subjected to occlusion, with leukocyte-endothelial cell adhesive interactions quantified at time points comparable to those described for mice subjected to 45 min of intestinal ischemia followed by 70 min reperfusion (Group 2, below).

***Group 2: I/R alone.*** Mice in this group (n=6) were treated as described for Group 1 above except that I/R was induced by occlusion of the superior mesenteric artery for 45 min followed by reperfusion for 70 min. Leukocyte rolling and adhesion were quantified during min 30-40 and 60-70 of reperfusion.

***Groups 3, 4, and 5: NaHS + I/R.*** In order to determine whether $H_2S$ can act as a preconditioning stimulus and prevent I/R-induced LR and LA, a solution of NaHS was used as a $H_2S$ donor and given at 24 hours prior to I/R (***NaHS(24 hr) + I/R, Group 3***) (late phase preconditioning), 1 hour prior to I/R (***NaHS(1 hr) + I/R, Group 4***) (early

phase preconditioning), or during reperfusion (***I/R + NaHS(sf), Group 5***). NaHS (Sigma Chemical, St. Louis, MO) was diluted in saline to produce a concentration of 1.4 mM and administered as a bolus at the lowest dose that is physiologically relevant and reported in the literature to deliver $H_2S$ to the intestine (n=6,14 μmol/kg i.p., volume of solution injected was 0.3 mL/30g mouse).

***Group 6: L-NIO + NaHS(24hr) + I/R.*** To determine whether the effects of late phase $H_2S$-PC to prevent postischemic leukocyte rolling and adhesion were initiated by an NO-dependent signaling mechanism, mice were treated with the specific, but non-isoform selective NOS inhibitor L-NIO. Mice in this group (n=3) were treated as described for Group 3 except that L-NIO (100 mg/kg i.p.) was administered 10 minutes prior to NaHS on Day 1, then subsequently exposed to I/R 24 hrs later (Day 2). We have previously used this L-NIO dosing protocol to demonstrate a role for NO as a trigger for late phase preconditioning induced by ethanol ingestion or adenosine (24, 53, 55).

**Groups 7-9: *Sham (eNOS -/-*** ), ***I/R (eNOS -/-), NaHS(24hr) + I/R (eNOS -/-).*** Because L-NIO is a specific, but non-isoform selective NOS inhibitor, we repeated the studies outlined for Groups 1-3 above in eNOS-deficient mice (*Sham (eNOS -/-* ), *I/R (eNOS -/-), NaHS + I/R (eNOS -/-), respectively*) to determine whether eNOS was the isoform responsible for triggering $H_2S$-PC.

***Group 10: SB203580 + NaHS(24hr) + I/R and Group 11: SK86002 + NaHS(24hr) + I/R:*** To determine whether the effects of late phase $H_2S$-PC were initiated by a p38 MAPK-dependent signaling mechanism, mice were treated with two different p38 MAPK inhibitors, SB203580 or SK86002. Mice in these groups (n=6 per group) were treated

as described for Group 6 except that the p38 MAPK inhibitors (10 mg/kg i.p.) , rather than L-NIO, were administered 10 minutes prior to NaHS.

*Statistical Analysis:* The data were analyzed with standard statistical analysis, i.e., ANOVA with Sheffe's (post hoc) test for multiple comparisons. All values are expressed as means ± SEM. Statistical significance was defined at $P < 0.05$.

## RESULTS

Figure 2 illustrates the average numbers of rolling (Upper Panel) and adherent (Lower Panel) leukocytes in postcapillary venules of the murine small intestine subjected to I/R alone (I/R, Group 2) or exposing the intestine to the $H_2S$ donor either 1 hr (NaHS(1hr) + I/R, Group 3) or 24 hrs NaHS(24hr) + I/R, Group 4) prior to I/R, or only during reperfusion (I/R + NaHS(sf), Group 5) relative to non-ischemic controls (Sham, Group 1). I/R induced marked increases in the numbers of rolling and adherent leukocytes after 30 and 60 min of reperfusion, proadhesive effects that were largely abolished by preconditioning with the $H_2S$ donor, NaHS, when the donor was given 24 h prior to I/R (NaHS(24hr) + I/R, Group 3). Interestingly, the protective effects of $H_2S$ were not elicited when the donor is given 1 hr prior to I/R (NaHS(1hr) + I/R, Group 4) (acute phase preconditioning) or when the intestine was exposed to the $H_2S$ donor via the superfusate during reperfusion (I/R + NaHS(sf), Group 5). These results indicate that NaHS treatment can induce late, but not early phase preconditioning and is ineffective in reducing leukocyte rolling and adhesion when administered during reperfusion.

The insets at the bottom to Figures 3 and 5 illustrate the time course for the appearance phosphorylated eNOS and p38 MAPK in representative western blots obtained in endothelial cells exposed to NaHS. NaHS exposure was associated with the appearance of phospho-eNOS and phospho-p38 MAPK within 1 and 10 min, peaking at 1-6 hrs and returning towards control values by 24 hrs. These observations led us to postulate that NaHS exposure may trigger the acquisition of delayed or late phase tolerance to I/R by activating eNOS and p38 MAPK. To address the role of

eNOS, we conducted two groups of studies. In the first, postischemic LR and LA were quantified in WT mice treated with the NOS inhibitor L-NIO coincident with NaHS administration on Day. NOS inhibition attenuated the effect of antecedent $H_2S$ to prevent postischemic LR (Figure 3, top panel). NOS inhibition only marginally influenced the effect of NaHS-PC to reduce LA after 30 min of reperfusion but was associated with a marked increase in LA at 60 min (Figure 3, bottom panel). To further explore this question, we examined the effectiveness of NaHS-PC in eNOS-deficient mice. I/R increased LR and LA in eNOS-/- mice to levels (Figure 4, top and bottom panels, respectively) that were similar to that noted in WT animals (Figure 3, top and bottom panels, respectively). Preconditioning with the $H_2S$ donor failed to limit postischemic leukocyte rolling in eNOS-deficient mice (Figure 4, top panel), a result which supports the concept that $H_2S$-dependent eNOS activation is essential for the development of this anti-inflammatory effect in I/R. However, the ability of NaHS-PC to prevent postischemic leukocyte adhesion persisted in eNOS-/- mice (Figure 5, bottom panel). Taken together with NOS inhibition data presented in Figure 3, the results obtained in eNOS-deficient mice (Figure 4) indicates that the effect of antecedent NaHS to limit postischemic leukocyte rolling occurs by an eNOS-dependent mechanism. However, the role of eNOS in the effect of NaHS to prevent stationary leukocyte adhesion is less prominent and suggests that other NOS isoforms (which are inhibited by L-NIO) may contribute to this latter effect or that compensatory alterations occur in eNOS-deficient mice.

To determine whether p38 MAPK also participates as an initiator of the anti-inflammatory actions elicited by $H_2S$ donor treatment, we evaluated the effects of two

different p38 MAPK inhibitors, SB203580 or SK86002, administered just prior to NaHS on LR and LA induced by I/R 24 hrs later (Figure 5, Upper and Lower Panels, respectively). Both inhibitors attenuated the effectiveness of NaHS as a preconditioning stimulus to reduce postischemic LR and LA.

## DISCUSSION

The results of this study provide the first evidence that exposing the small bowel to the $H_2S$ donor NaHS induces the development of an anti-inflammatory phenotype in murine small intestine such that postcapillary venules fail to support leukocyte rolling and adhesion when subjected to ischemia/reperfusion (I/R) 24 hrs later. Interestingly, treatment with NaHS 1 hr prior to I/R or by continuous superfusion with this donor during reperfusion failed to elicit these anti-adhesive responses. These observations suggest that $H_2S$ is effective at inducing delayed acquisition of tolerance to I/R (late phase preconditioning) but does not instigate early phase preconditioning nor does it exert a direct effect to limit postischemic leukocyte rolling and adhesion when the bowel is exposed to this donor during reperfusion. Our results contribute to a growing body of evidence indicating that $H_2S$, the third and most recently discovered endogenous gaseous signaling molecules, can be protective in the vascular system.

These observations are important because I/R is now well-recognized as one form of acute inflammation in which leukocytes play a key role (35). Recognition of the importance of the inflammatory process to the pathogenesis of I/R injury has led to an intensive research effort directed at identifying strategies to prevent leukocyte infiltration into postischemic tissues. Indeed, work conducted over the past 20 years has led to the development of the concept that oxidant-induced leukocyte/endothelial cell interactions are largely responsible for the microvascular dysfunction induced by reperfusion (18-20, 38, 40). Preconditioning with $H_2S$ donors may represent a promising new avenue for prevention of the microvascular complications of I/R.

In view of the powerful anti-adhesive effects of NaHS treatment 24 hrs prior to I/R, but not when administered 1 hr prior to induction of ischemia or during reperfusion, we focused our attention on the mechanisms that may be involved in eliciting late phase preconditioning by this $H_2S$ donor. Because it has been demonstrated that exposing the mesentery to exogenous $H_2S$ attenuates leukocyte rolling and adhesion induced by proinflammatory stimuli such as TNF$\alpha$ (16, 51), it is possible that the protective effects noted in the present study were due to the continued presence of sulfides 24 hrs after administration. However, this explanation is highly unlikely because of the inherent instability of $H_2S$ owing to oxidation in mitochondria, methylation in the cytosol, and scavenging by metalloproteins, disulfide-containing proteins, and heme compounds, which limits its biologic half-life . In addition, $H_2S$ is subject to rapid catabolism via thiol S-methyltransferase and rhodenese, both of which are expressed in the small intestine . Finally, exposing the small bowel to the NaHS during reperfusion failed to prevent postischemic LR and LA, results which suggest that the continued presence of the $H_2S$ donor during reperfusion is not required for its anti-adhesive induced by preconditioning 24 hrs earlier.

The aforementioned observations suggest that the protective actions of $H_2S$ preconditioning are initiated by activation of downstream signaling mechanisms, in a manner analogous to other short-lived preconditioning stimuli, such as adenosine and NO. Two distinct mechanisms for triggering the development of this protective effect were implicated by our studies. First, the effect of late phase NaHS-PC to prevent the postischemic increases in leukocyte rolling appears to be triggered by an eNOS-dependent mechanism. Second, inhibition of p38 MAPK coincident with NaHS

administration attenuated the effectiveness of this $H_2S$ donor in preventing leukocyte rolling and adhesion noted on exposure to I/R 24 hrs later, suggesting a role for this MAPK in late phase NaHS-PC.

A growing body of evidence indicates that $H_2S$ exerts a variety of effects that may limit I/R-induced injury and inflammation. For example, $H_2S$ produces vasorelaxation by activating $K_{ATP}$ channels (8, 15, 21, 26, 48, 51, 61, 62) and can enhance the vasodilatory effects of NO (21, 60), actions that may improve tissue perfusion in I/R. In addition, this gaseous signaling molecule reduces mitochondrial respiration, which may conserve ATP levels in ischemic tissues (57). $H_2S$ also exerts antioxidant effects and limits oxidative stress by virtue of its actions to raise intracellular glutathione levels by enhancing the activity of γ-glutamylcysteine synthetase and upregulating cysteine transport (28). Additionally, $H_2S$ enhances the ability of superoxide dismutase to scavenge superoxide (43). Furthermore, $H_2S$ reduces apoptosis induced by inhibition of caspase-3 cleavage and p38 MAPK phosphorylation (42) which may contribute to potential infarct-sparing effects in I/R. Moreover, inhibition of endogenous $H_2S$ synthesis reduces leukocyte rolling velocity and increases leukocyte adherence to mesenteric postcapillary venules under baseline conditions while NaHS treatment prevents leukocyte-endothelial cell adhesive interactions following exposure to TNFα (14, 16, 58). The former results indicate that basal $H_2S$ production serves as an endogenous modulator of leukocyte/endothelial cell adhesive interactions while the latter observation indicates that $H_2S$ donors may be effective in preventing adhesive responses induced by proinflammatory mediators. In our study, mesenteric superfusion with NaHS during reperfusion failed to prevent postischemic LR and LA, even though

the doses used were similar to those used in the aforementioned studies showing this $H_2S$ donor prevented TNF$\alpha$-induced adhesion. These disparate results may be explained by the more complicated inflammatory milieu induced by I/R, which involves the generation of multiple chemotactic stimuli.

The first in vivo study that suggested a possible protective role of $H_2S$ in I/R was conducted by Jeon and Lee (22) in the liver, where they found that S-adenosylmethionine, an $H_2S$ precursor, protects against mitochondrial injury, prevented mitochondrial oxidant stress, and improved ischemia-induced hepatic energy metabolism after ischemia. Subsequent work provided more direct support for the concept that $H_2S$ itself can protect against ischemia/reperfusion injury and inflammation, in cardiac myocytes and gastric mucosa, respectively (6, 7, 14, 37). Pan et al. used an isolated rat ventricular myocyte preparation to show that endogenous $H_2S$ contributes to cardioprotection induced by metabolic inhibition preconditioning (37).

In light of the aforementioned observations, we hypothesized that exposing the small bowel to the $H_2S$ donor NaHS during reperfusion would limit the increases in leukocyte rolling and adhesion. However, this treatment protocol failed to prevent the proinflammatory effects of I/R (Figure 2). We then turned our attention to the postulate that antecedent NaHS treatment may induce the development of an anti-inflammatory phenotype on subsequent exposure to I/R 1 or 24 hrs later, similar to the biphasic protective actions (early and late phase preconditioning) elicited by ischemic preconditioning, ingestion of ethanol, or exposure to a variety of pharmacologic agents. Our results provide the first evidence that exogenous $H_2S$ may also induce protection in I/R by instigating the development of a preconditioned, anti-inflammatory phenotype

such that postcapillary venules fail to support leukocyte rolling and adhesion in tissues exposed to I/R 24 hrs after treatment with this gaseous signaling molecule (Figure 2). However, in contrast to the preconditioning stimuli listed above, antecedent NaHS induced the development of an anti-inflammatory phenotype only when administered 24 hrs, but not 1 hr prior to I/R. These results indicate that NaHS induces late, but not early, phase acquisition of tolerance to ischemia in the small intestine, a property unique to this form of preconditioning.

In previous studies (17, 24, 29, 45, 54, 55), we have demonstrated that a variety of late phase preconditioning stimuli, including antecedent ethanol ingestion, short bouts of ischemia, adenosine A2 receptor agonists, exogenous CGRP or bradykinin, and AMP-activated protein kinase (AMPK) activators, induce the development of an anti-inflammatory phenotype that is triggered by eNOS-dependent NO release (55). Although $H_2S$ does not appear to produce vasodilation by a NOS-dependent mechanism, $H_2S$ can enhance the vasorelaxant effects of NO (21) and, conversely, the production of $H_2S$ can be up-regulated by NO (50, 51, 60). Thus, we evaluated the role of eNOS in the vasculoprotection afforded by late phase $H_2S$-PC by employing a pharmacologic inhibitor approach in WT animals and by use of an eNOS knockout model. The ability of $H_2S$-PC to prevent postischemic leukocyte rolling was completely absent in eNOS-deficient mice and was markedly attenuated by NOS inhibition with L-NIO in wild-type animals (Figures 3 and 4, respectively). Although the reductions in LA induced by NaHS-PC were attenuated by coincident administration of L-NIO, a specific but non-isoform selective NOS inhibitor, NaHS-PC remained effective in eNOS-deficient mice. Taken together, these results suggest that other NOS isoforms may contribute to

the effects of the $H_2S$ donor to prevent postischemic LA or that compensatory alterations occur in eNOS-deficient mice which allow this anti-adhesive effect to remain operant. An additional explanation is suggested by our recent work demonstrating that preconditioning with the AMPK agonist, 5-aminoimidazole-4-carboxamide 1-β-D-furanoside (AICAR), also prevents postischemic leukocyte rolling and adhesion, but eNOS appeared to be involved only in the effect of this agent on leukocyte rolling, but not leukocyte adhesion. The similarity in responses suggests the possibility that $H_2S$-PC may signal through AMPK to produce its salutary effect on leukocyte rolling. Clearly, much additional work will be required to evaluate this intriguing hypothesis.

The disparate findings with regard to the effect NaHS-PC in the presence of NOS inhibition vs eNOS deficiency were surprising in view of our earlier work demonstrating that the prevention of both leukocyte rolling and adhesion was abrogated by pharmacologic NOS inhibition and was absent in eNOS-/- mice in animals preconditioned by antecedent ethanol ingestion, short bouts of ischemia, exogenous CGRP or bradykinin, and adenosine A2 receptor agonists 24 hrs prior to I/R (24, 32, 55). In addition, preconditioning with NO donors also prevented both leukocyte rolling and adhesion during I/R in both WT and eNOS-deficient mice. The latter result is important because it indicates that the signaling mechanisms that are induced by NO downstream from eNOS activation remain intact in eNOS-/- mice.

Recent work indicates that the activation of a number of kinases, such as p38 MAPK, plays a critical role in the development of preconditioned states (3, 4, 23). At least 6 different isoforms of p38 MAPK have been described. This MAPK is activated by dual phosphorylation on a Thr-Gly-Tyr motif in response to a number of stimuli

implicated as triggers for preconditioning, including antecedent exposure to short bouts of ischemia or NO donors (29, 36) (10, 31, 33). Since $H_2S$ has been shown to increase p38 MAPK phosphorylation (56), we postulated that the anti-inflammatory effects induced by exogenous $H_2S$ might be elicited through activation of this kinase. Our results show that pharmacologic inhibition of p38 MAPK by either of SB203580 or SK80062 abolished the protective effects of late phase NaHS-PC to limit leukocyte rolling and leukocyte adhesion induced by I/R 24 hrs after preconditioning (Figure 6).

It is unclear whether $H_2S$ directly activates eNOS and/or p38 MAPK or targets other molecular elements upstream from these signaling enzymes. $H_2S$ can induce dithiol reduction and ligand displacement from heme iron (30, 44), posttranslational protein modifications that may subserve this function. In addition, $H_2S$ can interact with the oxygen binding site of cytochrome c oxidase in the respiratory chain, leading to enhanced mitochondrial production of reactive oxygen species (ROS) (13). The latter possibility is particularly appealing since ROS have been implicated in the triggering mechanism for other preconditioning stimuli, such as ethanol and short bouts of ischemia (12, 54). This is also interesting in light of our recent demonstration of a role for $K_{ATP}$ channels in NaHS-PC (unpublished observations), which can be activated by ROS.

In summary, our results provide the first evidence that treatment with NaHS, an exogenous source of $H_2S$, induces the development of an anti-inflammatory phenotype in postcapillary venules, preventing both postischemic LR and LA when given 24 h prior to I/R. Since the protection afforded by NaHS-PC on postischemic LR was prevented by NOS inhibition and was absent in eNOS-deficient mice, it appears that eNOS activation

triggers the appearance of this protective effect. However, the role of eNOS activation in the effect of NaHS-PC to limit postischemic leukocyte adhesion appears to be less prominent. On the other hand, NaHS-dependent p38 MAPK activation may play an essential role in preventing the increases in both leukocyte rolling and adhesion induced by subsequent exposure to I/R 24 hrs later.

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## FIGURE LEGENDS

**Figure 1. Experimental Protocols.** Illustration of the experimental protocols assigned to each group. The numbers along the top of the diagram in minutes refer to the time line of the protocol on Day 1, 24 hours prior to ischemia and reperfusion (I/R), and Day 2, the day of I/R. Hatched bars indicate digital video recording (10 min). Solid black bars indicate the 45 min period of ischemia during which the superior mesenteric artery had no blood flow. Triangles indicate administration of drug. See text for further details.

**Figure 2. Temporal effects NaHS-PC**. Preconditioning with the $H_2S$ donor NaHS 1 hr (NaHS-PC(1hr) + I/R) or 24 hrs (NaHS-PC(24hr) + I/R) prior to intestinal ischemia/reperfusion (I/R) on postischemic leukocyte rolling (Upper Panel) and adhesion (Lower Panel) vs superfusion of the bowel during I/R with NaHS (I/R + NaHS (sf)) relative to sham (no NaHS, no I/R) controls or I/R alone. * indicates mean values that were statistically different from the sham control group at $p<0.05$.

**Figure 3. Effects of late-phase NaHS-PC in WT mice.** Groups evaluated included ischemia/reperfusion alone (I/R) or I/R following pretreatment with a $H_2S$ donor (NaHS(24hr) + I/R), or NOS inhibition just prior to treatment with $H_2S$ donor (L-NIO + NaHS(24hr) + I/R) 24 hrs prior to I/R on postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion. * indicates mean values that were statistically different from the sham control group at $p<0.05$. Inset at the bottom depicts a representative western blot for

phosporylated eNOS at different time points following exposure to $H_2S$ donor, NaHS, in HMEC-1 cells.

**Figure 4. Effects of late-phase NaHS-PC in eNOS ko mice.** NaHS was given 24 hrs prior to ischemia/reperfusion (I/R) (NaHS-PC(24hr) + I/R) in on postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion eNOS -/- mice relative to sham control (no NaHS, no I/R) or I/R alone. * indicates mean values that were statistically different from the sham control group at $p<0.05$.

**Figure 5. Effects of p38 MAPK inhibition on the anti-adhesive effects of NaHS-PC.** Groups include sham controls (no NaHS, no I/R), ischemia/reperfusion alone (I/R), NaHS-PC(24hr) + I/R, or I/R plus treatment with a p38 MAPK inhibitors SB203580 (SB203580 + NaHS(24hr) + I/R) and SK80062 (SK80062 + NaHS(24hr) + I/R) 10 min prior to $H_2S$ donor preconditioning. Data include postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion. * indicates mean values that were statistically different from the $H_2S$ donor preconditioning group (NaHS(24hr) + I/R) at $p<0.05$. Inset at bottom depicts changes in phosphorylated p38 MAPK over time following exposure of HMEC-1 cells to 100 μM NaHS.

Figure 1

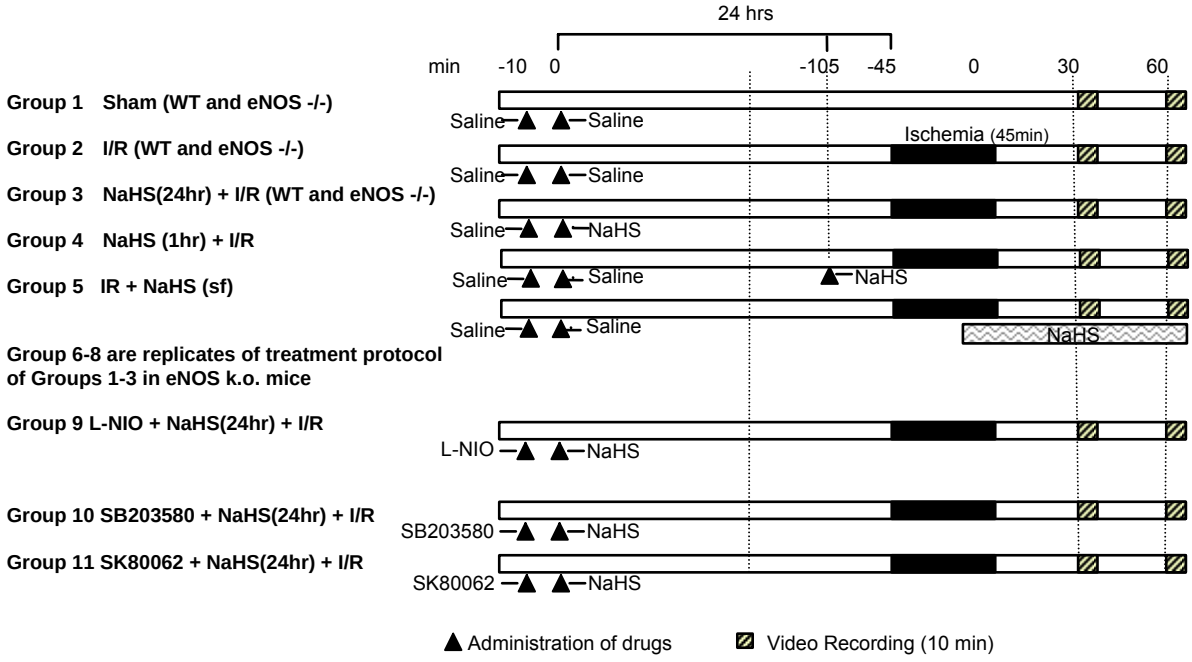
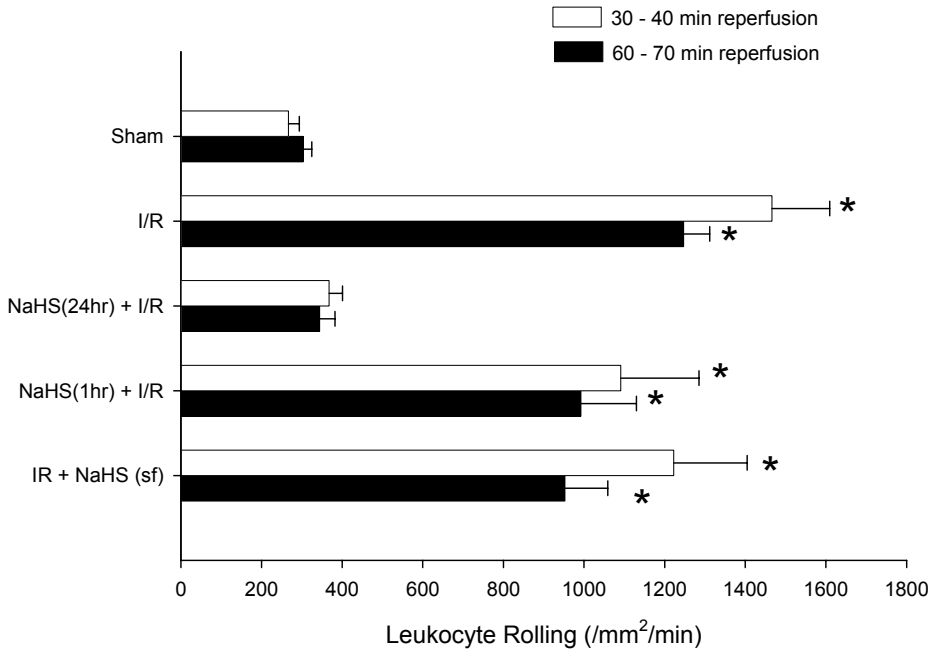

24 hrs
min
-10
0
-105
-45
0
30
60
Group 1 Sham (WT and eNOS -/-)
Saline
Saline
Group 2 I/R (WT and eNOS -/-)
Ischemia (45min)
Saline
Saline
Group 3 NaHS(24hr) + I/R (WT and eNOS -/-)
Saline
NaHS
Group 4 NaHS (1hr) + I/R
Saline
Saline
NaHS
Group 5 IR + NaHS (sf)
Saline
Saline
NaHS
Group 6-8 are replicates of treatment protocol of Groups 1-3 in eNOS k.o. mice
Group 9 L-NIO + NaHS(24hr) + I/R
L-NIO
NaHS
Group 10 SB203580 + NaHS(24hr) + I/R
SB203580
NaHS
Group 11 SK80062 + NaHS(24hr) + I/R
SK80062
NaHS
Administration of drugs
Video Recording (10 min)

Figure 2


30 - 40 min reperfusion
60 - 70 min reperfusion
Sham
I/R
NaHS(24hr) + I/R
NaHS(1hr) + I/R
IR + NaHS (sf)
*
0
200
400
600
800
1000
1200
1400
1600
1800
Leukocyte Rolling (/mm2/min)

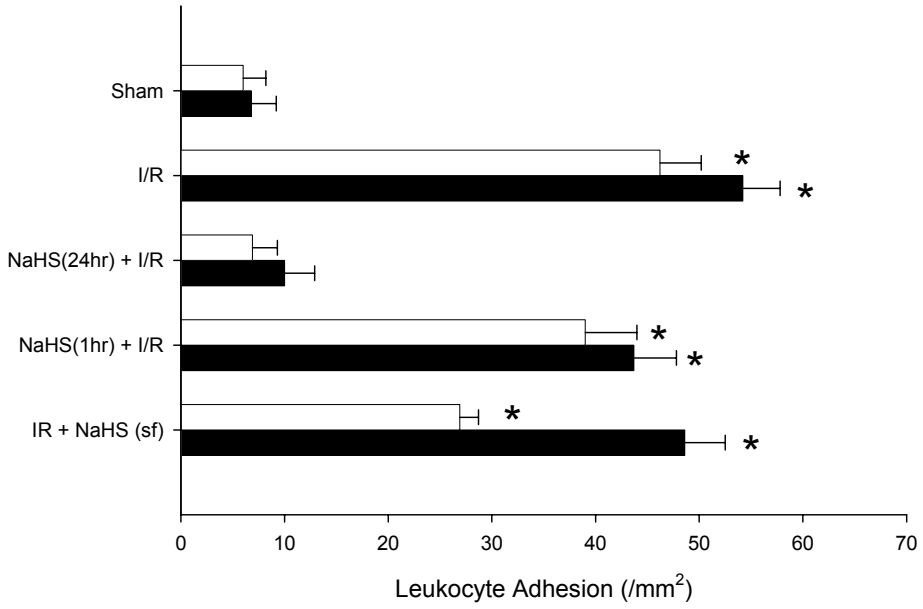
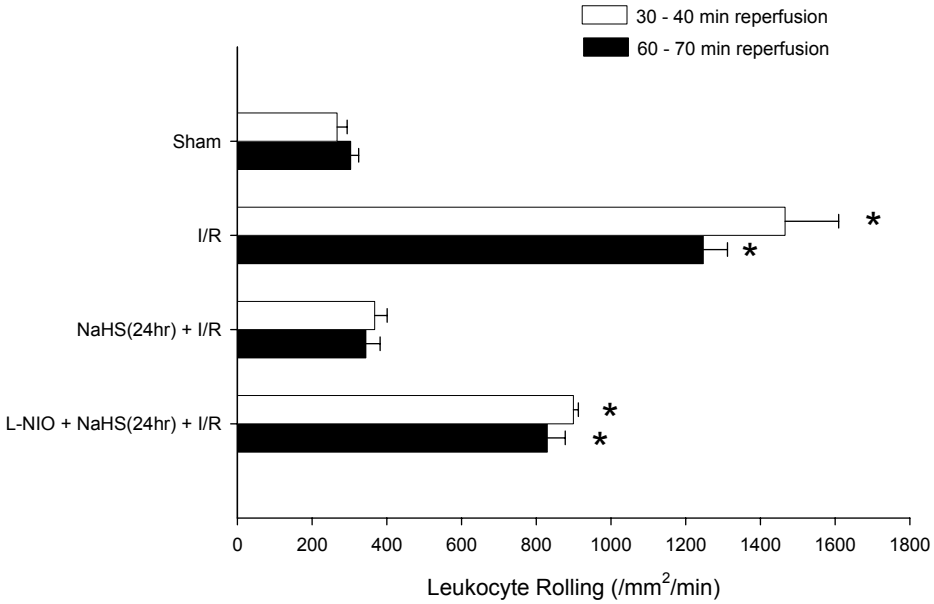
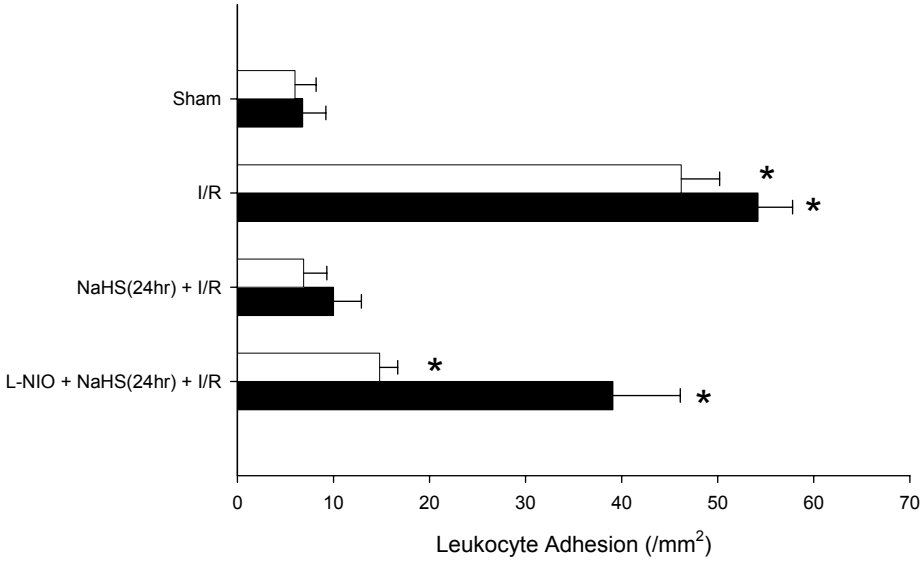

Sham
I/R
NaHS(24hr) + I/R
NaHS(1hr) + I/R
IR + NaHS (sf)
*
0
10
20
30
40
50
60
70
Leukocyte Adhesion (/mm2)

Figure 3




NaHS exposure 0” 1” 5” 10” 30” 1’ 6’ 24’

Figure 4

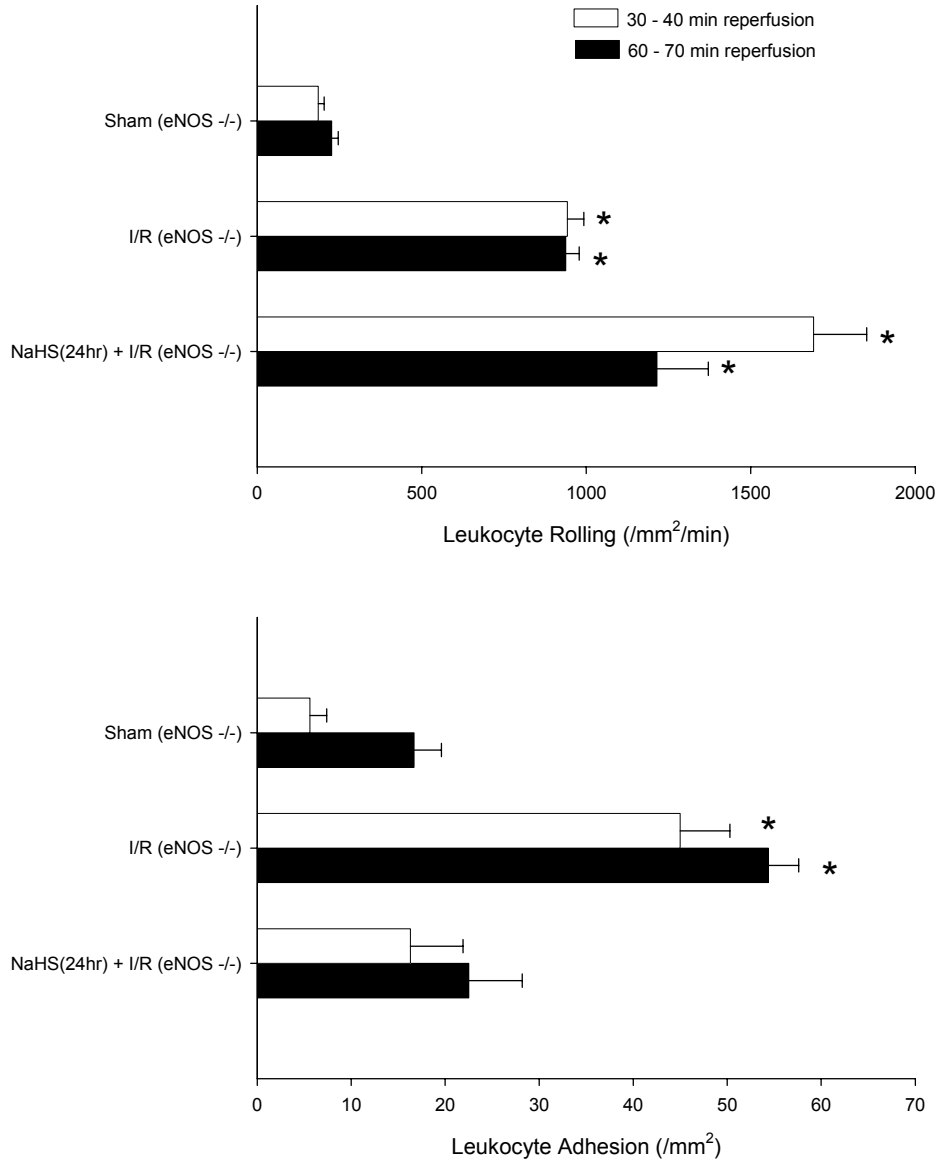
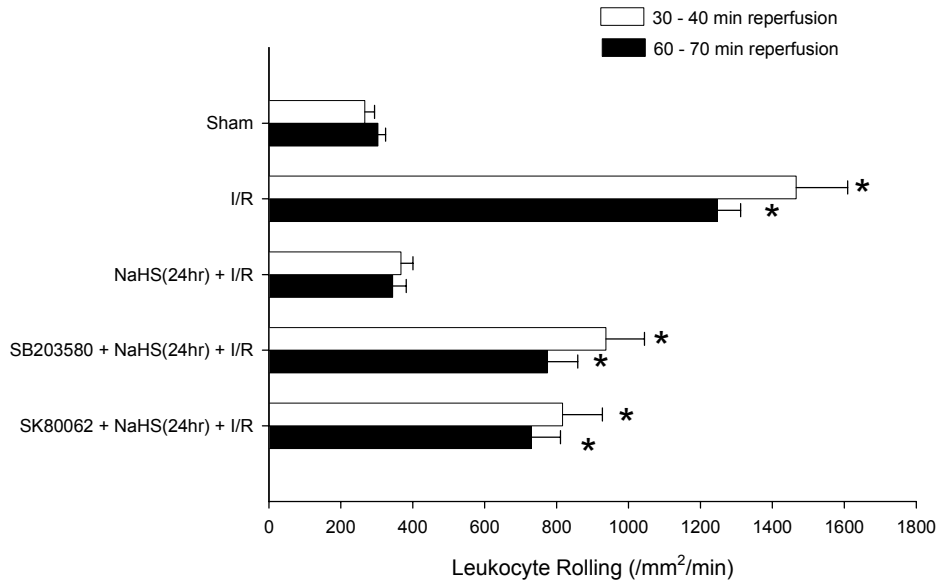
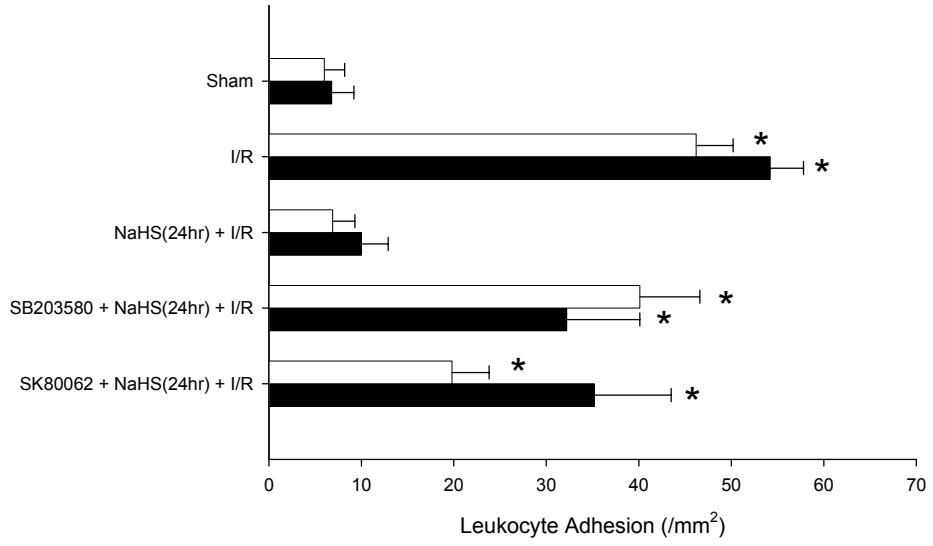

30 - 40 min reperfusion
60 - 70 min reperfusion
Sham (eNOS -/-)
I/R (eNOS -/-)
NaHS(24hr) + I/R (eNOS -/-)
*
*
*
*
0
500
1000
1500
2000
Leukocyte Rolling (/mm2/min)
Sham (eNOS -/-)
I/R (eNOS -/-)
NaHS(24hr) + I/R (eNOS -/-)
*
*
0
10
20
30
40
50
60
70
Leukocyte Adhesion (/mm2)

Figure 5




NaHS exposure 0” 1” 5” 10” 30” 1’ 6’ 24’

# ANTECEDENT HYDROGEN SULFIDE ELICITS AN ANTI-INFLAMMATORY PHENOTYPE IN POSTISCHEMIC MURINE SMALL INTESTINE: ROLE OF $K_{ATP}$ CHANNELS

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*Running head:* Hydrogen sulfide preconditioning

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## ABSTRACT

Hydrogen sulfide ($H_2S$) is one of many endogenous gases that exerts powerful effects in the cardiovascular system. We recently demonstrated that preconditioning with an exogenous $H_2S$ donor (NaHS-PC) 24 hrs prior to ischemia and reperfusion (I/R) causes postcapillary venules to shift to an anti-inflammatory phenotype in C57Bl/6 wild-type mice such that these vessels fail to support LR and LA during reperfusion. Since $H_2S$ induces vasodilation by opening of ATP-sensitive potassium ($K_{ATP}$) channels and these channels have been implicated in other forms of preconditioning, we employed a pharmacologic inhibitor approach to determine whether the anti-inflammatory effects induced by NaHS-PC also relies on opening of $K_{ATP}$ channels. I/R produced a marked increase in LR and LA, effects that were largely abolished by antecedent treatment with NaHS 24 hrs earlier. The postischemic anti-inflammatory effects of NaHS-PC were attenuated by glibenclamide (non-selective $K_{ATP}$ channel inhibitor) treatment coincident with NaHS, 24 hrs prior to I/R. To determine the role of mitochondrial vs plasmalemmal $K_{ATP}$ channels, separate groups of mice were treated with 5-hydroxydecanoate (5HD, a selective inhibitor of mitochondrial $K_{ATP}$ channels) or HMR-1098 (a selective inhibitor of plasmalemma $K_{ATP}$ channels) and NaHS 24 hrs prior to I/R. Both agents were effective in preventing the anti-inflammatory effects of NaHS-PC. In addition, preconditioning with a plasmalemmal-specific $K_{ATP}$ channel activator (MCC134) was effective in attenuating postischemic LR and LA.



**Keywords:** ischemia, reperfusion, leukocyte rolling, leukocyte adhesion, preconditioning

## INTRODUCTION

The recent discovery of hydrogen sulfide ($H_2S$) as the third endogenous gaseous signaling molecule is interesting with regard to a potential role as a preconditioning stimulus for a number of reasons. $H_2S$ is enzymatically produced by the vasculature through the actions of cystathionine $\gamma$-lyase (CSE, also referred to as cystathionase) and is present in the blood at concentrations in the range of 10-100 $\mu$M, levels that far exceed those reported for nitric oxide (NO) and carbon monoxide (CO). Like NO and CO, $H_2S$ produces vasorelaxation, but does so by a mechanism distinct from these gaseous monoxides, activating $K_{ATP}$ channels rather than guanylyl cyclase (1, 2, 12, 19, 20). However, $H_2S$ can enhance the vasorelaxant effects of NO (5) and, conversely, the production of $H_2S$ can be up-regulated by NO (18). We have recently showed that $H_2S$ can be given exogenously (NaHS) to yield a protected phenoytype by late-phase preconditioning that involves both eNOS and p38 MAPK. Because $H_2S$ induces vasodilation by activating $K_{ATP}$ channels, the purpose of this study was to determine whether late phase NaHS-PC may trigger entrance into an anti-inflammatory preconditioned state by a $K_{ATP}$ channel-dependent mechanism.

## MATERIALS AND METHODS

*Animals:* Wild-type (WT) male C57BL/6J mice (6-7 weeks of age) were obtained from the Jackson Laboratories (Bar Harbor, ME). All mice were maintained on standard mouse chow and used at 8-12 weeks of age. The experimental procedures described herein were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri – Columbia Institutional Animal Care and Use Committee.

*Surgical Procedures and Induction of I/R:* The mice were anesthetized initially with the mixture of ketamine (150 mg/kg body wt, i.p.) and xylazine (7.5 mg/kg body wt, i.p.). The right carotid artery was cannulated and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery catheter. Systemic blood pressure was recorded continuously with a personal computer (Power Macintosh 8600; Apple) equipped with an analog-to-digital converter (MP 100; Biopac Systems). Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE, Molecular Probes, Eugene, OR, USA) was dissolved in DMSO at a stock concentration of 5 mg/ml, divided into 25 µl aliquots, and stored in light-tight containers at -20 °C until further dilution immediately prior to intravenous injection. The left jugular vein was cannulated for administration of CFDA-SE. After these procedures, a midline abdominal incision was performed, the superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (sham) or 45 min. After the ischemic period, the clip was gently removed and leukocytes were labeled with CFDA-SE by intravenous administration of the fluorochrome solution (250 µg/ml saline) at 20 µl/min for 5 min. During the preparation, storage, and administration of CFDA-SE, care was taken to minimize light

exposure. Leukocyte/endothelial cell adhesive interactions were quantified over min 30-40 and 60-70 of reperfusion, as described below.

*Intravital Fluorescence Microscopy:* The mice were positioned on a 20 × 30-cm Plexiglas board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 × 3-cm hole centered in the Plexiglas. The exposed small intestine was superfused with warmed (37 °C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min using a peristaltic pump (Model M312; Gilson). The exteriorized region of the small bowel was covered with BBS-soaked gauze to minimize the tissue dehydration, temperature changes, and the influence of respiratory movements. The superfusate was maintained at 37 ± 0.5 °C by pumping the solution through a heat exchanger warmed by a constant-temperature circulator (Model 1130; VWR). Body temperature of the mouse was maintained between 36.5 and 37.5 °C by use of a thermostatically controlled heat lamp. The board was mounted on the stage of an inverted microscope (Diaphot TMD-EF; Nikon) and the intestinal microcirculation was observed through a 20× objective lens. Intravital fluorescence images of the microcirculation (excitation wavelength, 420-490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics) and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD; Sony) and recorded on digital video using a digital video recorder (DMR-E50; Panasonic) for off-line quantification of measured variables during playback

of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function onto the monitor.

The intravital microscopic measurements described below were obtained over minutes 30-40 and 60-70 of reperfusion or at equivalent time points in the control groups. The intestinal segment was scanned from the oral to aboral section and 10 single, unbranched venules (20-50 μm diameter, 100 μm length) were observed, each for 30 sec. Leukocyte-endothelial cell interactions (the numbers of rolling and firmly adherent leukocytes) were quantified in each of the 10 venules, followed by calculation of the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move or detach from the venular wall for a period equal to or greater than 30 sec. Rolling cells are defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than centerline velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adherent leukocytes were normalized by expressing each as the number of cells per $mm^2$ vessel area.

*Experimental Protocols:* The general design of the experimental protocols for each group in the study is described below (Figure 1).

***Group 1: Sham.*** As a time control for the effects of experimental duration, the mesentery of each mouse in this group (n=6) was superfused with bicarbonate-buffered saline. The superior mesenteric artery was exposed but not subjected to occlusion, with leukocyte-endothelial cell adhesive interactions quantified at time points comparable to those described for mice subjected to 45 min of intestinal ischemia followed by 70 min reperfusion (Group 2, below).

***Group 2: I/R alone.*** Mice in this group (n=6) were treated as described for Group 1 above except that I/R was induced by occlusion of the superior mesenteric artery for 45 min followed by reperfusion for 70 min. Leukocyte rolling and adhesion were quantified during min 30-40 and 60-70 of reperfusion.

***Group 3: NaHS + I/R.*** In order to determine whether $H_2S$ can act as a preconditioning stimulus and prevent I/R-induced LR and LA, a solution of NaHS was used as a $H_2S$ donor. NaHS (Sigma Chemical, St. Louis, MO) was weighed and diluted in saline at a concentration of 1.4 mM). Mice in this group (n=6) were treated as described for Group 2 except that NaHS (14 μmol/kg i.p., volume injected was 0.3ml/30g mouse) was administered 24 h prior to I/R.

***Group 4: Glibenclamide + NaHS + I/R.*** To further explore the triggering mechanisms underlying the development of the anti-inflammatory phenotype induced by $H_2S$-PC, we also investigated the role of $H_2S$-dependent $K_{ATP}$ channel activation. Initial investigation was carried out by treating mice with the selective $K_{ATP}$ channel inhibitor, glibenclamide (6 mg/kg i.p.). Glibenclamide (Sigma Chemical, St. Louis, MO) inhibits both plasmalemmal and mitochondrial $K_{ATP}$ channels. Mice in this group (n=6) were treated as described for Group 3 except that glibenclamide was administered 10 minutes prior to NaHS.

***Group 5: 5HD + NaHS + I/R.*** To further investigate the subtype of the $K_{ATP}$ channels triggering NaHS-PC, mice were treated with the mitochondrial specific $K_{ATP}$ channel inhibitor, 5-hydroxydecanoate (5-HD) (Sigma Chemical, St. Louis, MO). Mice in this group (n=7) were treated as described in Group 3 except that they received 5-HD (10 mg/kg i.p.) 5-10 minutes prior to NaHS. Because of the short half-life of 5-HD, great

care and attention was given to the exact timing of the dosing of 5-HD prior to NaHS, at least 5 minutes, but no greater than 10 minutes prior to NaHS.

***Group 6: HMR-1098 + NaHS + I/R.*** In order to determine whether plasmalemmal $K_{ATP}$ channel activation also contributes to NaHS-PC, mice were treated with the plasmalemmal-specific $K_{ATP}$ channel inhibitor, HMR-1098. HMR-1098 was obtained by generous gift from Dr. Garrett Gross (through Aventis Pharaceuticals, Germany). Mice in this group (n=7) were treated as described in Group 3, except that they received HMR-1098 (6 mg/kg i.p.) 10 minutes prior to NaHS.

***Group 7: MCC-134 + I/R.*** In order to determine whether activation plasmalemmal-specific $K_{ATP}$ channels could independently act as a preconditioning stimulus in the murine small intestine, mice were treated MCC-134, a dual pharmacophore that acts to activate sarcolemmal $K_{ATP}$ channels while inhibiting mitochondrial $K_{ATP}$ channels. This compound was obtained by generous gift from Mitsubishi Pharma, Japan. Mice in this group (n=6) were treated as described in Group 2 except that they received MCC-134 (30 μM x 0.5 mL i.p.) 24 hours prior to I/R.

*Statistical Analysis:* The data were analyzed with standard statistical analysis, i.e., ANOVA with Sheffe's (post hoc) test for multiple comparisons. All values are expressed as means ± SEM. Statistical significance was defined at $P < 0.05$.

## RESULTS

Figure 2 illustrates the average numbers of rolling (Upper Panel) and adherent (Lower Panel) leukocytes in postcapillary venules of the murine small intestine exposed to I/R alone (I/R, Group 2) or $H_2S$ donor 24 h prior to I/R (NaHS + I/R, Group 3) relative to non-ischemic controls (Sham, Group 1). I/R induced marked increases in the numbers of rolling and adherent leukocytes after 30 and 60 min of reperfusion, proadhesive effects that were abolished by preconditioning with the $H_2S$ donor, NaHS.

To investigate the triggering mechanism involved in the development of this anti-inflammatory phenotype in response to antecedent $H_2S$, postischemic LR and LA were quantified in WT mice treated with 3 different $K_{ATP}$ channel inhibitors that either block both plasmalemmal and mitochondrial $K_{ATP}$ channels (glibenclamide) (Figure 2) or selectively inhibit only mitochondrial (5-HD) or sarcolemmal (HMR-1098) $K_{ATP}$ channels (Figure 3). All three inhibitors markedly reduced the protective effect elicited by $H_2S$ donor treatment to limit postischemic leukocyte rolling and leukocyte adhesion. Thus, $K_{ATP}$ channel activation secondary to NaHS treatment appears to play a role in preventing I/R-induced leukocyte rolling and leukocyte adhesion. Interestingly, preconditioning with the selective sarcolemmal $K_{ATP}$ channel activator, MCC-134, in lieu of $H_2S$, was effective in attenuating I/R-induced leukocyte rolling and adhesion. However, preconditioning with this agent was not as effective as NaHS-PC at returning LR and LA back to Sham levels (Figure 4).

## DISCUSSION

The results of this study provide evidence of two related mechanisms for triggering the development of the protective effects of late phase NaHS-PC. First, the effect of NaHS-PC to prevent the postischemic increases in leukocyte rolling and adherence appears to be triggered by both mitochondrial and plasmalemmal $K_{ATP}$ channel-dependent mechanisms. Moreover, our data show that preconditioning can be accomplished in the murine small intestine by activation of the plasmalemmal specific $K_{ATP}$ channels with MCC134. Our data provides new insights into pharmacologic therapy that may be beneficial to ischemia/reperfusion injury syndromes, suggesting that $H_2S$ donors may effectively precondition tissues to resist the proinflammatory effects of I/R.

A growing body of evidence indicates that $H_2S$ exerts a variety of effects that may limit I/R-induced injury and inflammation. For example, $H_2S$ produces vasorelaxation by activating $K_{ATP}$ channels (1, 3, 5, 7, 12, 19, 20) and can enhance the vasodilatory effects of NO (5, 18), actions that may improve tissue perfusion in I/R. In addition, this gaseous signaling molecule reduces mitochondrial respiration, which may conserve ATP levels in ischemic tissues. Recent evidence has shown that inhibition of endogenous $H_2S$ synthesis reduces leukocyte rolling velocity and increases leukocyte adherence to mesenteric postcapillary venules under baseline conditions. The latter results indicate that $H_2S$ production serves as an endogenous modulator of leukocyte/endothelial cell adhesive interactions. While the aforementioned results suggest that H2S may be effective in reducing I/R injury when applied during the ischemic insult, the results of the present study (Figure 2) and our earlier work

(manuscript under review) provide the first evidence that $H_2S$ may also induce protection in I/R by instigating the development of a preconditioned, anti-inflammatory phenotype such that postcapillary venules fail to support leukocyte rolling and adhesion in tissues exposed to I/R 24 hrs after treatment with this gaseous signaling molecule.

In earlier work, we have demonstrated that a variety of preconditioning stimuli, including antecedent ethanol ingestion, short bouts of ischemia, adenosine A2 receptor agonists, exogenous CGRP or bradykinin, and AMP-activated protein kinase (AMPK) activators induce the development of an anti-inflammatory phenotype that is triggered by NO formed by eNOS (6, 14-16). We recently demonstrated that $H_2S$ donor treatment 24 hrs prior to I/R also induced precondition by an eNOS-dependent mechanism (manuscript under review). Since the vasodilator actions of $H_2S$ are mediated by activation of $K_{ATP}$ channels and antecedent treatment with $K_{ATP}$ channel openers exerts infarct-sparing effects, prevents capillary no-reflow, and attenuates neutrophil infiltration in tissues subsequently exposed to I/R (1, 4, 8, 10, 13, 17), we hypothesized that entrance into the anti-inflammatory phenotype induced by $H_2S$ preconditioning might be initiated by activation of potassium channels. To address this postulate, we first treated mice with $K_{ATP}$ channel inhibitor glibenclamide, which effectively abolished the effect of $H_2S$-PC to prevent postischemic leukocyte rolling and leukocyte adhesion (Figure 2). These observations suggest that $K_{ATP}$ channel activation induced by $H_2S$-PC selectively target the molecular events that mediate postischemic leukocyte rolling and leukocyte adhesion.

While our observations with glibenclamide support a role for $K_{ATP}$ channels as a trigger for the development of $H_2S$-PC, this agent inhibits both plasmalemmal and

mitochondrial $K_{ATP}$ channels. To determine which of these $K_{ATP}$ channels might play a more dominant role in the development of the anti-inflammatory phenotype in response to antecedent treatment with $H_2S$, we first evaluated the effects of the selective mitochondrial $K_{ATP}$ channel inhibitor, 5-HD (Figure 3). In accordance with the effects of glibenclamide, 5-HD also prevented the protective effects of $H_2S$-PC to prevent leukocyte rolling and adhesion. These results suggest that mitochondrial $K_{ATP}$ channels play a role in triggering the effect of $H_2S$-PC to limit postischemic leukocyte rolling and adhesion. Next, we evaluated the role plasmalemmal $K_{ATP}$ channels in the development of these postischemic anti-inflammatory effects by treating mice with HMR-1098, a highly selective inhibitor of plasmalemmal $K_{ATP}$ channels, coincident with NaHS (Figure 3). Like glibenclamide and 5-HD, this agent attenuated the effects of NaHS-PC to limit postischemic leukocyte rolling and leukocyte adhesion. In addition, we demonstrated that preconditioning with a selective sarcolemmal $K_{ATP}$ channel opener MCC-134 was effective in preventing postischemic leukocyte rolling and adhesion (Figure 4). Because 5-HD and HMR-1098 were no more effective than glibenclamide, it appears that combined treatment with selective mitochondrial vs plasmalemmal $K_{ATP}$ channel antagonists would be no more effective than with either agent alone.

Overall, our results support a role for activation of both mitochondrial and plasmalemmal $K_{ATP}$ channels as an important step in instigating the development of the effects of NaHS-PC to prevent leukocyte rolling and adhesion induced by I/R 24 hrs later. Our results provide the first evidence that NaHS induces the development of an anti-inflammatory phenotype in postcapillary venules through both mitochondrial and plasmalemmal-specific channel activation.

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## FIGURE LEGENDS

**Figure 1.** Illustration of the experimental protocols assigned to each group. The numbers along the top of the diagram in minutes refer to the time line of the protocol on Day 1, 24 hours prior to ischemia and reperfusion (I/R), and Day 2, the day of I/R. Hatched bars indicate digital video recording (10 min). Solid black bars indicate the 45 min period of ischemia during which the superior mesenteric artery had no blood flow. Triangles indicate administration of drug. See text for further details.

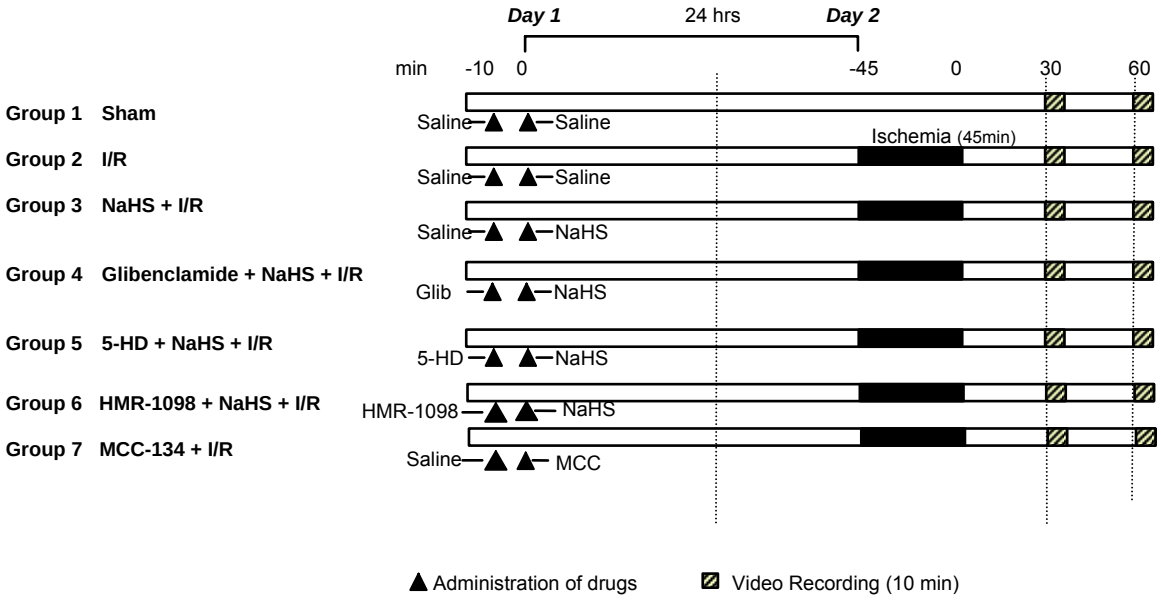
**Figure 2.** Effects of ischemia/reperfusion alone (I/R) or I/R following pretreatment with a $H_2S$ donor (NaHS + I/R), or $K_{ATP}$ channel inhibition just prior to treatment with $H_2S$ donor (Glib + NaHS +I/R) on postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion. * indicates mean values that were statistically different from the NaHS + IR group at $p<0.05$.

**Figure 3.** Effects of mitochondrial and plasmalemmal-specific $K_{ATP}$ channel activation as a trigger of the $H_2S$-PC stimulus. Groups include ischemia/reperfusion alone (I/R) or I/R plus treatment with a $K_{ATP}$ channel inhibitor 10 min prior to $H_2S$ donor preconditioning. Specific $K_{ATP}$ channel inhibition was achieved using mitochondrial specific $K_{ATP}$ channel inhibitor, 5-hydroxydecanoate (5-HD + NaHS + I/R) or plasmalemmal specific KATP channel inhibitor, HMR-1098 (HMR1098 + NaHS + I/R). Data include postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion. * indicates mean

values that were statistically different from the $H_2S$ donor preconditioning group (NaHS + I/R) at $p<0.05$.

**Figure 4.** Effects of plasmalemmal $K_{ATP}$ channel activation as a preconditioning stimulus. Plasmalemmal specific $K_{ATP}$ channel activator (MCC-134) was given 24h prior to I/R. * indicates mean values that were statistically different from the $H_2S$ donor preconditioning group (NaHS + I/R) at $p<0.05$.

Figure 1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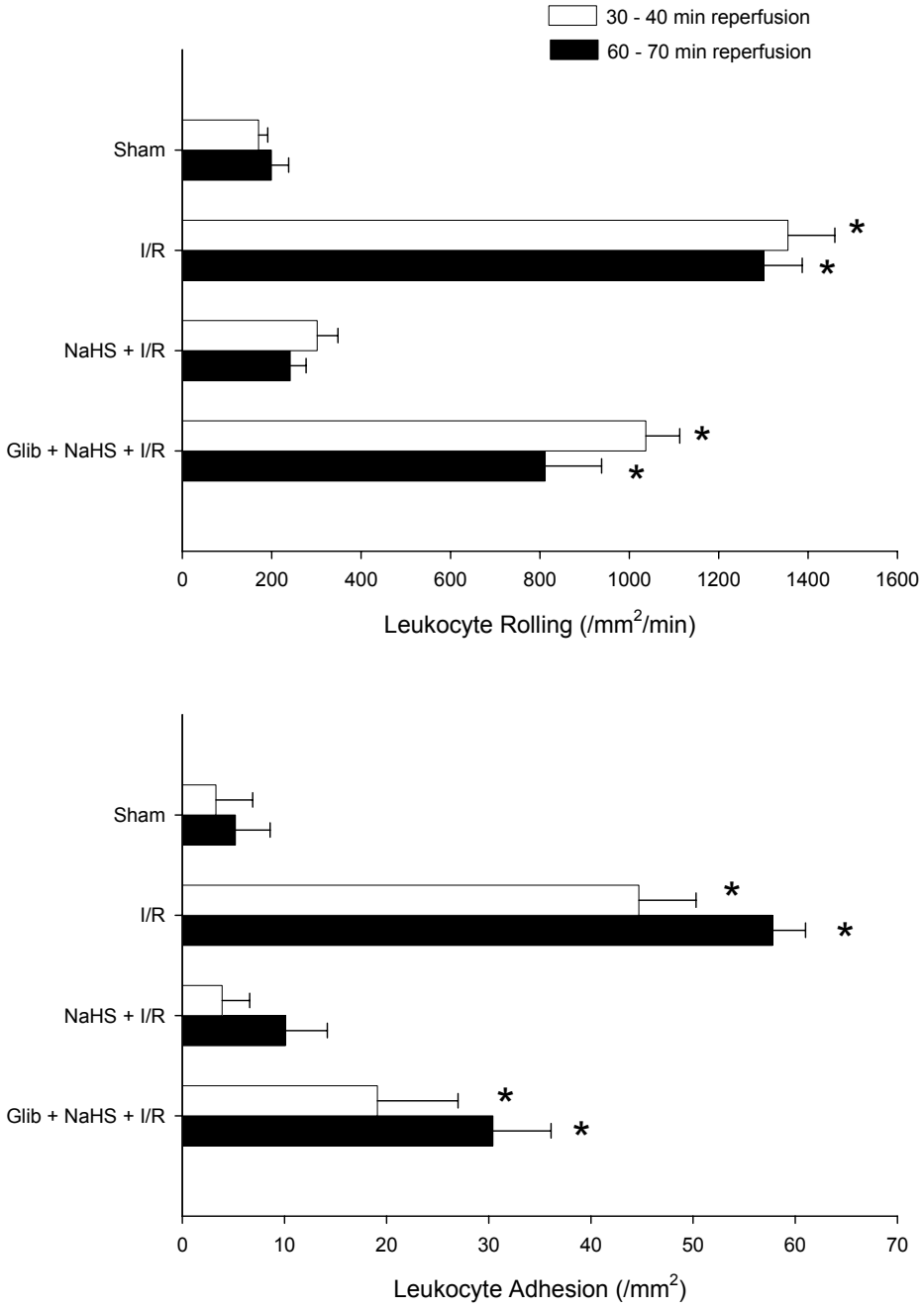
24 hrs
Day 2
min
-10
0
-45
0
30
60
Group 1 Sham
Saline
Saline
Ischemia (45min)
Group 2 I/R
Saline
Saline
Group 3 NaHS + I/R
Saline
NaHS
Group 4 Glibenclamide + NaHS + I/R
Glib
NaHS
Group 5 5-HD + NaHS + I/R
5-HD
NaHS
Group 6 HMR-1098 + NaHS + I/R
HMR-1098
NaHS
Group 7 MCC-134 + I/R
Saline
MCC
Administration of drugs
Video Recording (10 min)

Figure 2


30 - 40 min reperfusion
60 - 70 min reperfusion
Sham
I/R
NaHS + I/R
Glib + NaHS + I/R
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0
200
400
600
800
1000
1200
1400
1600
Leukocyte Rolling (/mm2/min)
Sham
I/R
NaHS + I/R
Glib + NaHS + I/R
*
0
10
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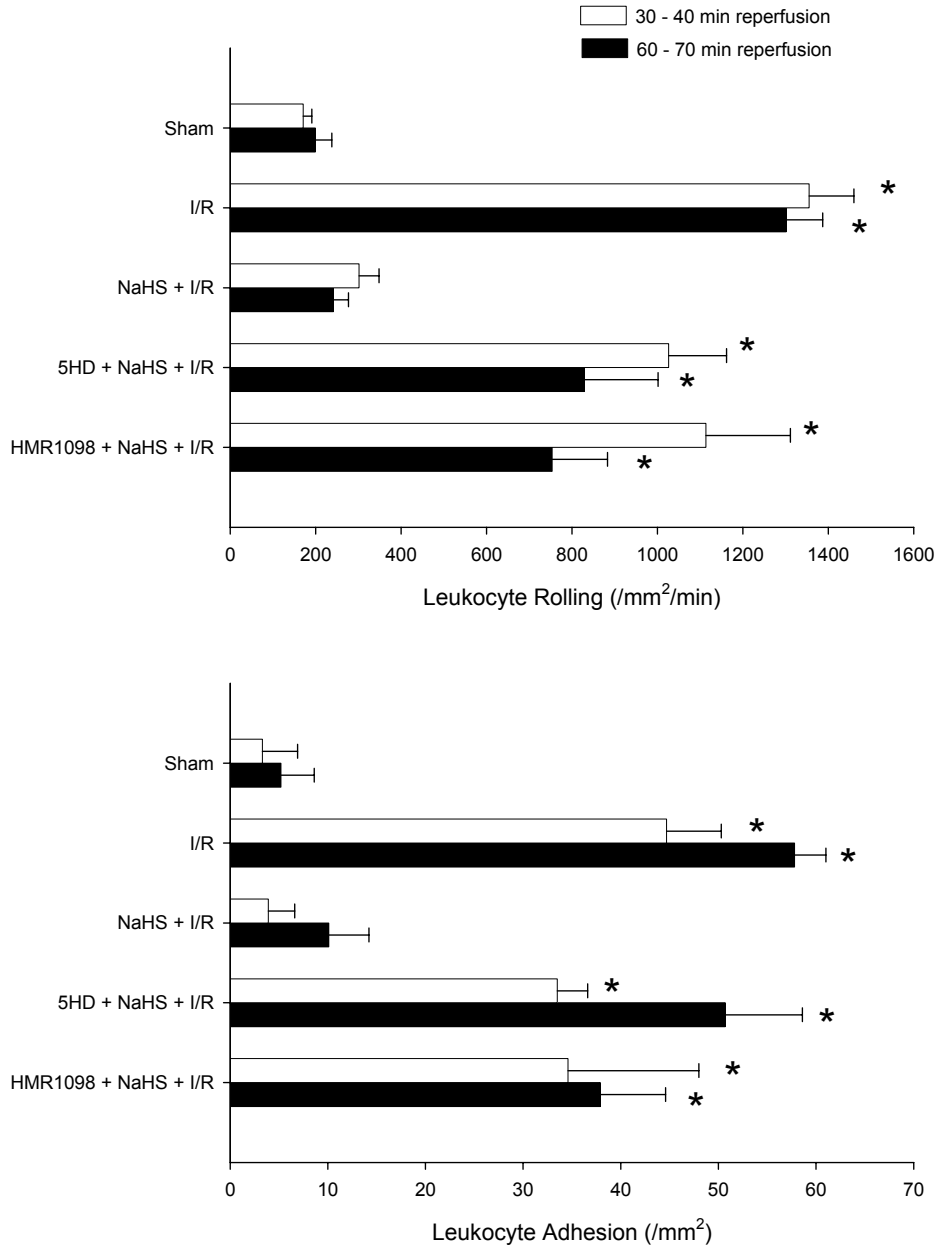
70
Leukocyte Adhesion (/mm2)

Figure 3


30 - 40 min reperfusion
60 - 70 min reperfusion
Sham
I/R
NaHS + I/R
5HD + NaHS + I/R
HMR1098 + NaHS + I/R
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200
400
600
800
1000
1200
1400
1600
Leukocyte Rolling (/mm2/min)
Sham
I/R
NaHS + I/R
5HD + NaHS + I/R
HMR1098 + NaHS + I/R
0
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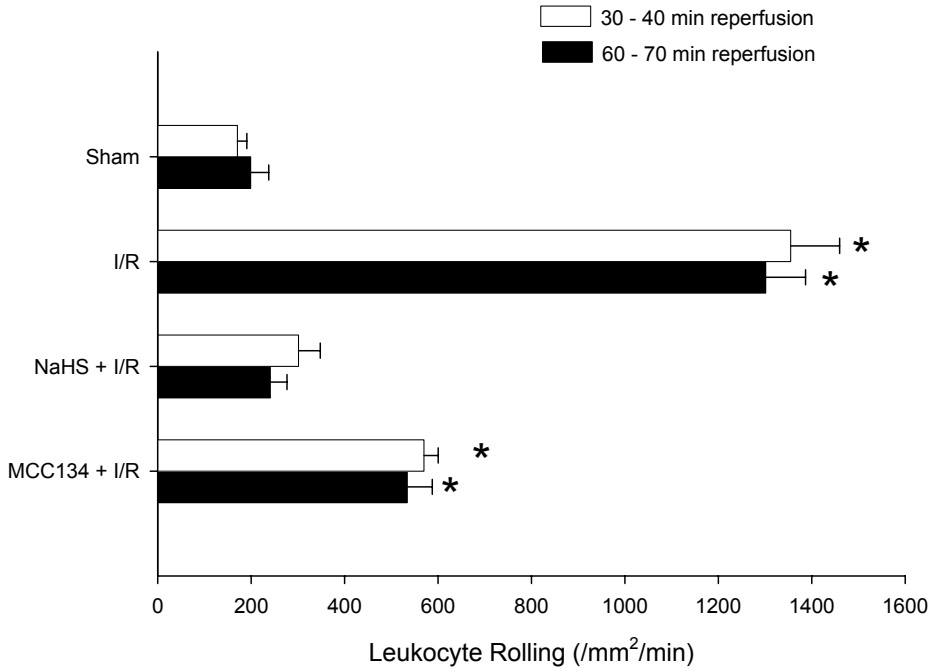
Leukocyte Adhesion (/mm2)

Figure 4


30 - 40 min reperfusion
60 - 70 min reperfusion
Sham
I/R
NaHS + I/R
MCC134 + I/R
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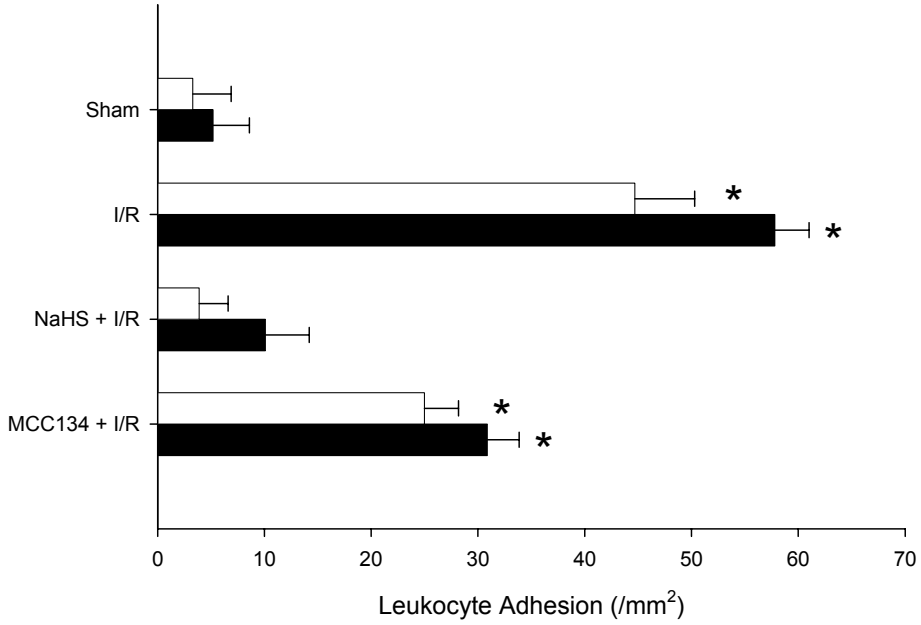
Leukocyte Rolling (/mm2/min)


Sham
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MCC134 + I/R
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Leukocyte Adhesion (/mm2)

# ANTECEDENT HYDROGEN SULFIDE ELICITS AN ANTI-INFLAMMATORY PHENOTYPE IN POSTISCHEMIC MURINE SMALL INTESTINE: ROLE OF $BK_{Ca}$ CHANNELS

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*Running head:* Hydrogen sulfide preconditioning: Activation of $BK_{Ca}$ channels

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## ABSTRACT

Hydrogen sulfide ($H_2S$) is one of many endogenous gases that exerts powerful effects in the cardiovascular system. We recently demonstrated that preconditioning with an exogenous $H_2S$ donor (NaHS-PC) 24 h prior to ischemia and reperfusion (I/R) causes postcapillary venules to shift to an anti-inflammatory phenotype in C57Bl/6 wild-type mice such that these vessels fail to support LR and LA during reperfusion. We have also previously shown that this pattern of $H_2S$-induced late-phase preconditioning is triggered by eNOS- and $K_{ATP}$ channel-dependent mechanisms. The objective of the present study was to determine whether the calcium-sensitive large conductance K channel ($BK_{Ca}$ channel) is also involved as an initiator of the anti-inflammatory phenotype elicited by $H_2S$. The postischemic anti-inflammatory effects of NaHS-PC were abolished by $BK_{Ca}$ channel inhibitor treatment (paxilline) when administered coincident with NaHS, 24 hrs prior to I/R. Moreover, preconditioning with the $BK_{Ca}$ channel activator, NS-1619, was as effective as NaHS-PC in preventing I/R-induced leukocyte rolling and adhesion. We also demonstrated that $H_2S$ directly activates $BK_{Ca}$ channels in cultured endothelial cells exposed to NaHS using patch-clamp techniques. The latter studies demonstrated that NaHS activates iberiotoxin-sensitive $K^+$ channels in endothelial cells and that this effect is not dependent on intracellular $Ca^+$ concentrations. Our data is consistent with the concept that H2S induces the development of an anti-adhesive state in I/R by a $BK_{Ca}$ channel-dependent mechanism.



**Keywords:** ischemia, reperfusion, leukocyte rolling, leukocyte adhesion, preconditioning

## INTRODUCTION

The recent discovery of hydrogen sulfide ($H_2S$) as the third endogenous gaseous signaling molecule is interesting with regard to a potential role as a preconditioning stimulus for a number of reasons. $H_2S$ is enzymatically produced by the vasculature through the actions of cystathionine $\gamma$-lyase (CSE, also referred to as cystathionase) and is present in the blood at concentrations in the range of 10-100 $\mu$M, levels that far exceed those reported for nitric oxide (NO) and carbon monoxide (CO). Like NO and CO, $H_2S$ produces vasorelaxation, but does so by a mechanism distinct from these gaseous monoxides, activating $K_{ATP}$ channels rather than guanylyl cyclase (5, 6, 33, 45, 46). However, $H_2S$ can enhance the vasorelaxant effects of NO (12) and, conversely, the production of $H_2S$ can be up-regulated by NO (44). The cross-talk between the gaseous signaling molecules is only beginning to be understood but is of great physiologic relevance in the microvasculature, with specific application to cardiovascular disease and ischemia/reperfusion syndromes.

We recently demonstrated that preconditioning with an exogenous $H_2S$ donor (NaHS-PC) 24 h prior to ischemia and reperfusion (I/R) causes postcapillary venules to shift to an anti-inflammatory phenotype in C57BL/6J wild-type mice such that these vessels fail to support LR and LA during reperfusion (Yusof et al**.,** in press). Moreover, we showed that the development of NaHS-PC is triggered by an eNOS-dependent mechanism. The latter observation, when coupled with recent work demonstrating that endothelial $BK_{Ca}$ (also termed Slo or MaxiK) channels play a role in regulating the synthesis of NO (13, 22, 31, 37), suggests the possibility that these potassium channels may also be involved in the genesis of the preconditioned anti-inflammatory phenotype

that develops in response to antecedent NaHS treatment. This notion is further supported by fact that preconditioning with the selective $BK_{Ca}$ opener NS1619 induces both early and late phase preconditioning in the myocardium, effects that were not blocked by $K_{ATP}$ channel antagonists but were mitigated by the selective $BK_{Ca}$ blockers, iberiotoxin or paxilline (28, 36, 38). In addition, the cardioprotective effects of estradiol appear to involve $BK_{Ca}$ channel activation rat ventricular myocytes exposed to simulated ischemia (19, 23). Importantly, endothelial cells do not express voltage-gated calcium channels, therefore, changes of intracellular calcium concentrations are due mainly to calcium release from internal stores or through transmembrane calcium influx, which is dependent of membrane hyperpolarization (22). Potassium channels have been shown to regulate the endothelial membrane potential and thereby influence intracellular calcium levels (22). For these reasons, we hypothesized that $H_2S$ donor treatment (NaHS-PC) elicits a preconditioned anti-adhesive phenotype via a $BK_{Ca}$ channel-dependent mechanism.

## MATERIALS AND METHODS

*Animals:* Wild-type (WT) male C57BL/6J WT mice (6-7 weeks of age) were obtained from the Jackson Laboratories (Bar Harbor, ME). All mice were maintained on standard mouse chow and used at 8-12 weeks of age. The experimental procedures described herein were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri – Columbia Institutional Animal Care and Use Committee.

*Surgical Procedures and Induction of I/R:* The mice were anesthetized initially with the mixture of ketamine (150 mg/kg body wt, i.p.) and xylazine (7.5 mg/kg body wt, i.p.). The right carotid artery was cannulated and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery catheter. Systemic blood pressure was recorded continuously with a personal computer (Power Macintosh 8600; Apple) equipped with an analog-to-digital converter (MP 100; Biopac Systems). Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE, Molecular Probes, Eugene, OR, USA) was dissolved in DMSO at a stock concentration of 5 mg/ml, divided into 25 µl aliquots, and stored in light-tight containers at -20 °C until further dilution immediately prior to intravenous injection. The left jugular vein was cannulated for administration of CFDA-SE. After these procedures, a midline abdominal incision was performed, the superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (sham) or 45 min. After the ischemic period, the clip was gently removed and leukocytes were labeled with CFDA-SE by intravenous administration of the fluorochrome solution (250 µg/ml saline) at 20 µl/min for 5 min. During the preparation, storage, and administration of CFDA-SE, care was taken to minimize light

exposure. Leukocyte/endothelial cell adhesive interactions were quantified over min 30-40 and 60-70 of reperfusion, as described below.

*Intravital Fluorescence Microscopy:* The mice were positioned on a 20 × 30-cm Plexiglas board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 × 3-cm hole centered in the Plexiglas. The exposed small intestine was superfused with warmed (37 °C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min using a peristaltic pump (Model M312; Gilson). The exteriorized region of the small bowel was covered with BBS-soaked gauze to minimize the tissue dehydration, temperature changes, and the influence of respiratory movements. The superfusate was maintained at 37 ± 0.5 °C by pumping the solution through a heat exchanger warmed by a constant-temperature circulator (Model 1130; VWR). Body temperature of the mouse was maintained between 36.5 and 37.5 °C by use of a thermostatically controlled heat lamp. The board was mounted on the stage of an inverted microscope (Diaphot TMD-EF; Nikon) and the intestinal microcirculation was observed through a 20× objective lens. Intravital fluorescence images of the microcirculation (excitation wavelength, 420-490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics) and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD; Sony) and recorded on digital video using a digital video recorder (DMR-E50; Panasonic) for off-line quantification of measured variables during playback

of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function onto the monitor.

The intravital microscopic measurements described below were obtained over minutes 30-40 and 60-70 of reperfusion or at equivalent time points in the control groups. The intestinal segment was scanned from the oral to aboral section and 10 single, unbranched venules (20-50 μm diameter, 100 μm length) were observed, each for 30 sec. Leukocyte-endothelial cell interactions (the numbers of rolling and firmly adherent leukocytes) were quantified in each of the 10 venules, followed by calculation of the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move or detach from the venular wall for a period equal to or greater than 30 sec. Rolling cells are defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than centerline velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adherent leukocytes were normalized by expressing each as the number of cells per $mm^2$ vessel area.

*Experimental Protocols:* The general design of the experimental protocols for each group in the study is described below.

***Group 1: Sham.*** As a time control for the effects of experimental duration, the mesentery of each mouse in this group (n=6) was superfused with bicarbonate-buffered saline. The superior mesenteric artery was exposed but not subjected to occlusion, with leukocyte-endothelial cell adhesive interactions quantified at time points comparable to those described for mice subjected to 45 min of intestinal ischemia followed by 70 min reperfusion (Group 2, below).

***Group 2: I/R alone.*** Mice in this group (n=6) were treated as described for Group 1 above except that I/R was induced by occlusion of the superior mesenteric artery for 45 min followed by reperfusion for 70 min. Leukocyte rolling and adhesion were quantified during min 30-40 and 60-70 of reperfusion.

***Group 3: NaHS + I/R.*** In order to determine whether $H_2S$ can act as a preconditioning stimulus and prevent I/R-induced LR and LA, a solution of NaHS was used as a $H_2S$ donor. NaHS (Sigma Chemical, St. Louis, MO) was weighed and diluted in saline at a concentration of 1.4 mM). Mice in this group (n=6) were treated as described for Group 2 except that NaHS (14 μmol/kg i.p., 0.3 mL) was administered 24 h prior to I/R.

***Group 4: Paxilline + NaHS + I/R.*** To explore the role of $BK_{Ca}$ channels as a trigger for the development of NaHS-PC, we investigated the effects of administration of the selective BK channel inhibitor, paxilline (25 mg/kg i.p.) (25) coincident with NaHS 24 hrs prior to I/r. Mice in this group (n=6) were treated as described for Group 3 except that paxilline was administered 10 minutes prior to NaHS.

***Group 5: NS-1619 + I/R.*** The aim of this group of experiments was to determine whether preconditioning with the $BK_{Ca}$ channel opener, NS-1619 (1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3H)benzimid-axolone), would mimic the effects of NaHS-PC and prevent postischemic LR and LA on subsequent exposure of the small intestine to I/R 24 hrs later. Mice in this group (n=6) were treated as described in Group 3 except that they received NS-1619 (100 μM**,** 0.5 mL i.p.) 24 hours prior to I/R in lieu of NaHS.

The final series of experiments in this study were directed at demonstrating that NaHS, at a concentration we achieved in our in vivo studies, could activate $BK_{Ca}$ in channels, using patch clamp techniques.

*HMEC-1 Culture*: Human microvascular endothelial cells (HMEC-1) were purchased from ATCC and cultured as described previously (1) and The cells were seeded and grown in MCDB-131 (Sigma Aldrich, St. Louis, MO) medium containing L-glutamine and sodium bicarbonate (11.6g/L), glucose and 10% (v/v) fetal bovine serum (Invitrogen), and maintained at 37 °C with 5% $CO_2$. The cells were grown to 80-90% confluency, then detached using 0.25% trypsin/0.5 mM EDTA in phosphate-buffered saline and reseeded onto sterile glass coverslips in 35 mm culture dishes. The cells allowed to attach to un-caoted coverslips and were incubated at $37^0$C with 5% $CO_2$ for one hour, and then the dishes were moved to a $28^0$C incubator just prior to electrophysiological recordings.

*Electrophysiological Recording*. A coverslip with cells was placed in a recording chamber (0.5 mL) mounted on the stage of an inverted microscope and a continuous perfusion was initiated. The patch clamp technique was employed with an EPC-9 amplifier (HEKA, Germany) to measure membrane currents in the whole-cell and inside-out configurations as described previously. The HMEC-1 cell line has been previously shown to have $BK_{Ca}$ channel activity (11). For the experiments in the whole-cell configuration, 3 mM Mg-ATP was included to inhibit ATP-sensitive K channels ($K_{ATP}$) and provide substrate for energy-dependent processes. The amplifier was controlled by a Dell XPS computer running Pulse + Pulsefit software through an ITC-16 interface (Instrutech, Port Washington, NY). Igor Pro (WaveMetrics, Oswego, OR) and SigmaPlot 9.0 (SPSS Inc.) were used for data analysis. Currents were filtered at 3.3

kHz and sampled at 10 kHz. Whole-cell $BK_{Ca}$ currents were activated by voltage step pulses delivered from a holding potential of -60 mV to potentials ranging from -80 to +80 mV in 10 mV increments, duration of 300 ms or voltage ramp pulse from -100 mV to +100 mV, duration of 1 s). Micropipettes were pulled from borosilicate glass capillaries (ID, 1.2 mm; OD, 1.5 mm; World Precision Instruments, Sarasota, FL) using a Sutter P-97 electrode puller and resistances ranged 3.0-3.5 MΩ when filled with a pipette solution. All experiments were performed at room temperature.

*Solutions.* For whole-cell recordings of HMEC-1 cells, the bath solution contained (in mM) 140 NaCl, 5.6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 15 Glucose, 10 Hepes, 2 Na-Pyruvate, pH 7.4, and pipette solution contained 140 KCl, 8 NaCl, 1 EGTA, 3 ATP-Mg, 10 Hepes, pH 7.2. A proper amount of a 0.1 M $CaCl_2$ solution was added to give a 600 nM free [$Ca^{2+}$]. A single BK channel current was recorded from an inside-out patch clamp configuration. The bath solution contained 140 KCl, 10 Hepes, 2 EGTA, 1 $MgCl_2$ (pH7.2 with KOH) and variable amounts of a 0.1 M $CaCl_2$ solution were added to give desired free [$Ca^{2+}$]. The level of free $Ca^{2+}$ in each solution was confirmed by using a calcium electrode (Orion model 93-20, WPI, Sarasota, FL). The pipette solution contained 140 KCl, 2 EGTA, 1.8 CaCl2, 10 Hepes, 1$MgCl_2$ (pH 7.4).

*Chemicals*. The $BK_{Ca}$ channel blocker iberiotoxin (IBTX) was obtained from Sigma-Aldrich (St. Louis, MO) and paxilline was obtained from Transduction Laboratories (Lexington, KY). All stocks were made in bath solution. 100nM IBTX, 10 μM paxilline, 100 μM NaHS were the final concentrations in the bath.

*Statistical Analysis:* The data were analyzed with standard statistical analysis, i.e., ANOVA with Sheffe's (post hoc) test for multiple comparisons. All values are expressed as means ± SEM. Statistical significance was defined at $P < 0.05$.

## RESULTS

Figure 2 illustrates the average numbers of rolling (Upper Panel) and adherent (Lower Panel) leukocytes in postcapillary venules of the murine small intestine exposed to I/R alone (I/R, Group 2) or $H_2S$ donor 24 h prior to I/R (NaHS + I/R, Group 3) relative to non-ischemic controls (Sham, Group 1). I/R induced marked increases in the numbers of rolling and adherent leukocytes after 30 and 60 min of reperfusion, proadhesive effects that were abolished by preconditioning with the $H_2S$ donor, NaHS.

To investigate the role of $BK_{Ca}$ channels in the triggering mechanism involved in the development of this anti-inflammatory phenotype in response to antecedent $H_2S$, postischemic LR and LA were quantified in mice treated with the $BK_{Ca}$ channel antagonist, paxilline, coincident with NaHS-PC 24 hrs prior to I/R (Figure 2). Paxilline effectively abolished the effects elicited by $H_2S$ donor treatment to limit postischemic leukocyte rolling and leukocyte adhesion. This result suggests that $BK_{Ca}$ channel activation secondary to NaHS treatment plays a crucial role in preventing I/R-induced leukocyte rolling and leukocyte adhesion. Interestingly, preconditioning with the selective $BK_{Ca}$ channel activator, NS-1619, in lieu of $H_2S$, was as effective as NaHS-PC in attenuating I/R-induced leukocyte rolling and adhesion, further supporting a role for $BK_{Ca}$ channel activation as a trigger for the development of an anti-inflammatory phenotype in I/R

To obtain direct evidence that NaHS could activate $BK_{Ca}$ channels, we used patch-clamp techniques to monitor the effect of this $H_2S$ donor activity on iberiotoxin-sensitive currents in cultured endothelial cells. HMEC-1 cells have previously been shown to express $BK_{Ca}$ channel activity (11), which we verified in our experiments (Figure 3). We

went on to demonstrate that $BK_{Ca}$ channel activity increases (about 50%) in HMEC-1 cells exposed to NaHS (Figure 4) and that this channel activity is sensitive to iberiotoxin, verifying that it is most probably $BK_{Ca}$ channel current. The current-voltage relations appear linear (-80 mV to +60 mV) in HMEC-1 cells under the inside-out configuration (Figure 5). Finally, these effects are similar at either 600 nM or 1 μM free calcium concentrations in the pipette solutions (Figure 6).

## DISCUSSION

A growing body of evidence indicates that $H_2S$ exerts a variety of effects that may limit I/R-induced injury and inflammation. For example, $H_2S$ produces vasorelaxation by activating $K_{ATP}$ channels (5, 8, 12, 16, 33, 45, 46) and can enhance the vasodilatory effects of NO (12, 44), actions that may improve tissue perfusion in I/R. In addition, this gaseous signaling molecule reduces mitochondrial respiration, which may conserve ATP levels in ischemic tissues (2, 3, 14, 17, 18, 21, 29, 30, 34). Recent evidence has shown that inhibition of endogenous $H_2S$ synthesis reduces leukocyte rolling velocity and increases leukocyte adherence to mesenteric postcapillary venules under baseline conditions (43). The latter results indicate that $H_2S$ production serves as an endogenous modulator of leukocyte/endothelial cell adhesive interactions. While the aforementioned results suggest that $H_2S$ may be effective in reducing I/R injury when applied during the ischemic insult, the results of the present study (Figure 2) and our earlier work (Yusof et al. AJP, manuscript under review) provide the first evidence that $H_2S$ may also induce protection in I/R by instigating the development of a preconditioned, anti-inflammatory phenotype such that postcapillary venules fail to support leukocyte rolling and adhesion in tissues exposed to I/R 24 hrs after treatment with this gaseous signaling molecule.

Potassium channels are ubiquitously expressed cell membrane proteins that participate in a wide variety of physiological processes, including regulation of vasomotor tone, heart rate, neurotransmitter release and muscle contraction. Given the broad range of functional activities, it is not surprising that a diverse spectrum of functionally distinct potassium channels have evolved. With regard to preconditioning,

ATP-sensitive potassium channels have received the most attention. However, the results of several recent studies point to the importance of another set of potassium channels, the large conductance, calcium-activated potassium ($BK_{Ca}$) channels, as critical triggers for the development of the protected phenotype in response to preconditioning stimuli. These channels are expressed by parenchymal cells in a variety of organs, but also in vascular tissues, including endothelial and vascular smooth muscle cells (10). $BK_{Ca}$ channels can be activated by elevations in intracellular calcium and by membrane depolarization and demonstrate single channel conductances of ~250 pS. $BK_{Ca}$ channels are comprised of a pore-forming $\alpha$-subunit and a modulatory $\beta$-subunit that is sensitive to channel blocking agents (10). In the heart, $BK_{Ca}$ channels are thought to present only in the mitochondria, whereas endothelial cells express these channels on the plasma membrane (10).

In earlier work, we have demonstrated that a variety of preconditioning stimuli, including antecedent ethanol ingestion, short bouts of ischemia, adenosine $A_2$ receptor agonists, exogenous CGRP or bradykinin, and AMP-activated protein kinase (AMPK) activators induce the development of an anti-inflammatory phenotype that is triggered by NO formed by eNOS (9, 15, 26, 40-42). We recently demonstrated that $H_2S$ donor treatment 24 hrs prior to I/R also induced preconditioning by an eNOS-dependent mechanism that also involves activation of $K_{ATP}$ channels (Yusof et al., AJP, manuscript under review). Due to the recently demonstrated cross-talk between $K_{ATP}$ channels and $BK_{Ca}$ channels, the present study is of particular interest. As we have already shown that the $H_2S$ donor elicits a preconditioned phenotype by activation of $K_{ATP}$ channels, we sought to determine whether BK channels also play a role in NaHS-PC.

The present study provides strong support for the notion that NaHS-PC activates $BK_{Ca}$ channels in endothelial cells, which play a critical role in the development of an anti-inflammatory phenotype in the murine small intestine, such that postcapillary venules fail to support increased leukocyte rolling and adhesion induced by exposing the bowel to I/R 24 hrs after application of this preconditioning stimulus. Three lines of evidence support this conclusion. First, coincident administration of the $BK_{Ca}$ channel inhibitor paxilline with NaHS 24 hrs prior to I/R effectively abolished the effects of NaHS-PC to prevent postischemic leukocyte rolling and adhesion. Second, the anti-inflammatory effects of NaHS-PC were mimicked by preconditioning with the $BK_{Ca}$ channels activator NS-1619. Third, we provided evidence that exposing cultured endothelial cells to NaHS at the same concentration achieved in our in vivo studies, activates an iberiotoxin-sensitive current.

A cardioprotective role for $BK_{Ca}$ channels was first suggested by O'Rourke and coworkers (10), who demonstrated that administration of NS1619 just prior to I/R reduced myocardial infarct size. The powerful infarct-sparing effects of $BK_{Ca}$ channel activation were prevented by paxilline, providing additional support for the notion that these specific channels limit infarct size. Subsequent work has demonstrated NS1619 induces both early and late phase preconditioning in myocardium, effects that were not blocked by $K_{ATP}$ channel antagonists but were mitigated by the selective $BK_{Ca}$ blockers, iberiotoxin or paxilline. In addition, the cardioprotective effects of estradiol appear to involve $BK_{Ca}$ channel activation in rat ventricular myocytes exposed to simulated ischemia. There is also evidence that the infarct sparing effects of early phase ischemic preconditioning may involve $BK_{Ca}$ channel activation. While not yet appreciated, our

results demonstrate that in addition to infarct-sparing effects in the myocardium, $BK_{Ca}$ channel activation also promotes the development of an anti-inflammatory phenotype that may reduce leukocyte-dependent reperfusion in injury. Our results also extend the notion that antecedent $BK_{Ca}$ channel activation produces protective effects in the small intestine in addition to the heart. Finally, we also demonstrate for the first time, the contribution of $BK_{Ca}$ channels in response to a novel preconditioning stimulus, NaHS-PC.

Our conclusions are largely based on the specificity of paxilline and NS1619 for $BK_{Ca}$ channels. However, it is known that these agents produce other effects. For example, paxilline has been shown to inhibit sarco/endoplasmic reticulum $Ca^{2+}$-ATPase both in vitro and in vivo (35, 36), although at concentrations higher than were likely achieved in our experiments. Iberiotoxin is a more specific $BK_{Ca}$ channel inhibitor, but use of this neurotoxin in vivo is precluded by expense. On the other hand, NS1619 can inhibit complex I of the mitochondrial respiratory chain in tumor cells (20) and modify mitochondrial membrane potential. This has potential implications for our studies since $H_2S$ has been reported to inhibit mitochondrial cytochrome oxidase, leading to oxidative stress. Since the generation of reactive oxygen species have been implicated as triggers for ischemic and ethanol preconditioning (40), it is possible that the anti-inflammatory state induced by NS1619 preconditioning may be related to oxidant production. Although the actions of NS1619 are not inhibited by $K_{ATP}$ channel antagonists, more recent work indicates that NS1619 stimulates $Ca^{2+}$-gated chloride channels (24) and there is some evidence that choride channels participate in ischemic preconditioning in the myocardium (4). Although we have not directly tested these

potential complicating side effects, our observation that NaHS activates $BK_{Ca}$ channels in endothelial cells, when coupled with the demonstration that paxilline blocks the protective actions of NS1619 in our model, argue in favor of the interpretation that NaHS-PC occurs by a $BK_{Ca}$ channel-dependent mechanism.

The mechanism underlying NaHS-dependent $BK_{Ca}$ channel activation is unclear as are the downstream signaling events that mediate NaHS-PC. However, $H_2S$ is known to elicit an oxidative stress secondary to its effect to inhibit cytochrome oxidase, which may activate $BK_{Ca}$ channels (7). Conversely, it has also been proposed that since mitochondrial $BK_{Ca}$ channel opening increases matrix $K^+$, which in turn alters mitochondrial matrix $H^+$ by $K^+/H^+$ exchange, thereby stabilizing mitochondrial membrane potential despite increased respiration, thereby enhancing the generation of superoxide (32). In light of our earlier work demonstrating a role for eNOS in the development of NaHS-PC (Yusof et al., AJP, manuscript under review) and the fact that $BK_{Ca}$ channels can regulate the synthesis of NO, it is tempting to speculate that NaHS-induced eNOS activation may be an early downstream event. However, Wang et al. have provided evidence that NS1619 produced myocardial preconditioning by a NOS-independent mechanism (36). We have obtained strong evidence that heme oxygenase-1 is an end-effector of the anti-inflammatory phenotype during I/R 24 hrs, a conclusion based on the observations that NaHS-PC is ineffective in wild-type mice treated with a heme oxygenase inhibitor and is absent in HO-1 knockout animals (Yusof et al., AJP, manuscript under review). However, the mechanistic links between $BK_{Ca}$ channel activation induced by NaHS and increased HO-1 activity during I/R 24 hrs later are

unknown. Clearly, much additional work will be required to answer these important questions.

In summary, the results of this study not only provide evidence for a novel triggering mechanism for the development of the anti-inflammatory effects induced by late phase NaHS-PC, but also introduce a potential new physiologic effect of endogenous $H_2S$. Specifically, the effect of NaHS-PC to prevent the postischemic increases in leukocyte rolling and adherence appears to be triggered by $BK_{Ca}$ channel-dependent mechanisms. Moreover, our data show that preconditioning can be accomplished by activation of the $BK_{Ca}$ channels with NS-1619 in the murine small intestine. In addition, our studies establish that NaHS can directly activate $BK_{Ca}$ channels in cultured endothelial cells. These observations provide new insight regarding the potential use of $BK_{Ca}$ channel activators and NaHS as rational therapeutic agents to limit leukocyte-dependent reperfusion injury in I/R and perhaps other inflammatory conditions. In addition, our results suggest the possibility that development of interventions directed at enhancing the endogenous production of $H_2S$ may be effective in limiting the inflammatory component of I/R injury.

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## FIGURE LEGENDS

**Figure 1.** Illustration of the experimental protocols assigned to each group. The numbers along the top of the diagram in minutes refer to the time line of the protocol on Day 1, 24 hours prior to ischemia and reperfusion (I/R), and Day 2, the day of I/R. Hatched bars indicate digital video recording (10 min). Solid black bars indicate the 45 min period of ischemia during which the superior mesenteric artery had no blood flow. Triangles indicate administration of drug. See text for further details.

**Figure 2.** Effects of BK channel inhibition on the preconditioning effects of NaHS. Effects of ischemia/reperfusion alone (I/R), I/R following pretreatment with a $H_2S$ donor (NaHS + I/R), or BK channel inhibition just prior to treatment with $H_2S$ donor (Paxilline + NaHS +I/R), and BK channel activation in lieu of NaHS (NS-1619 + I/R) on postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion (n=6 in all groups). * indicates mean values that were statistically different from the NaHS + IR group at $p<0.05$.

**Figure 3.** Iberiotoxin-sensitive channels are present in HMEC-1 cells. The whole cell outward current traces of HMEC-1 cells and shown under control (PBS) conditions (panel A), 1 min after iberiotoxin (IBTX) application (panel B), 3 min after IBTX application (panel C) and after washout of IBTX (panel D). The averaged current-voltage relations of outward currents of control (open circles), 1 min after IBTX (closed circles), 3 min after IBTX (closed box) and after washout (open box) are shown in panel

E. The schematic of the experimental setup is shown in panel F. All pipette solutions had ~600 nM free $Ca^{2+}$.

**Figure 4.** Single $K^+$ channel currents increase in HMEC-1 cells exposed to NaHS and are blocked by IBTX. The representative tracing shows a single K channel current of a cell exposed to control solution (PBS), NaHS (100 nM) or IBTX + NaHS (100 nM each). All pipette solutions contained 1 μM free calcium.

**Figure 5.** Voltage dependence of HMEC-1 $BK_{Ca}$ channel. Under the inside-out configuration (panel C), unitary channel currents were recorded at various membrane potentials (-80 mV to +60 mV) denoted at the bottom of each current trace shown in panel A. The summary of the current-voltage relations of $BK_{Ca}$ channels is shown in panel B. A schematic of the inside out configuration is shown in panel C. The pipette solutions contained 1 μM free calcium.

**Figure 6.** There is no effect of intracellular $Ca^{2+}$ concentrations on single $BK_{Ca}$ channel currents in HMEC-1 exposed to NaHS. A representative tracing of a single channel current in HMEC-1 cells exposed to control solution (PBS), NaHS, or IBTX + NaHS in the presence of 600 nM free calcium is shown in panel A. The corresponding (600 nM free calcium) graphical representation of each current normalized to control is shown in panel B. A representative tracing of a single channel current in HMEC-1 cells exposed to control solution (PBS), NaHS, or IBTX + NaHS in the presence of 1 μM free calcium

is shown in panel C. The corresponding (1 μM free calcium) graphical representation of each current normalized to control is shown in panel D.

Figure 1

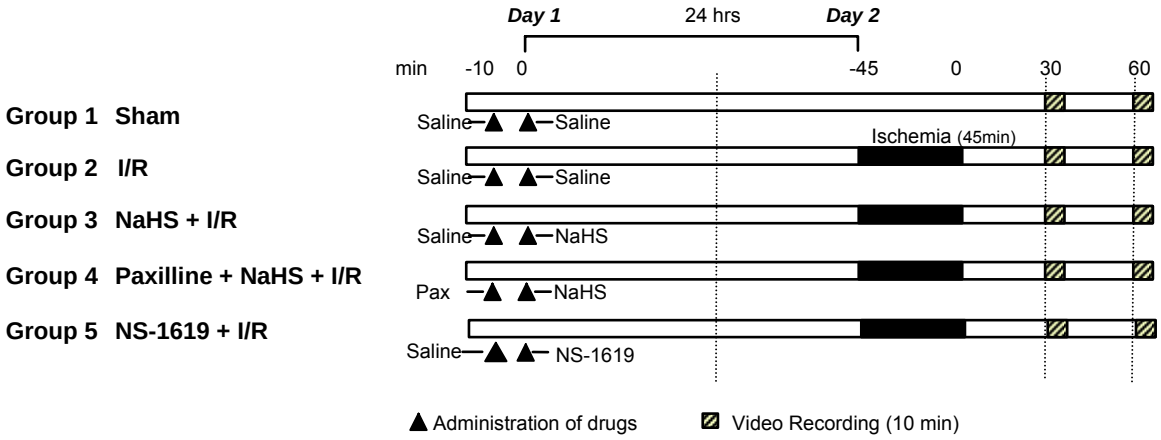
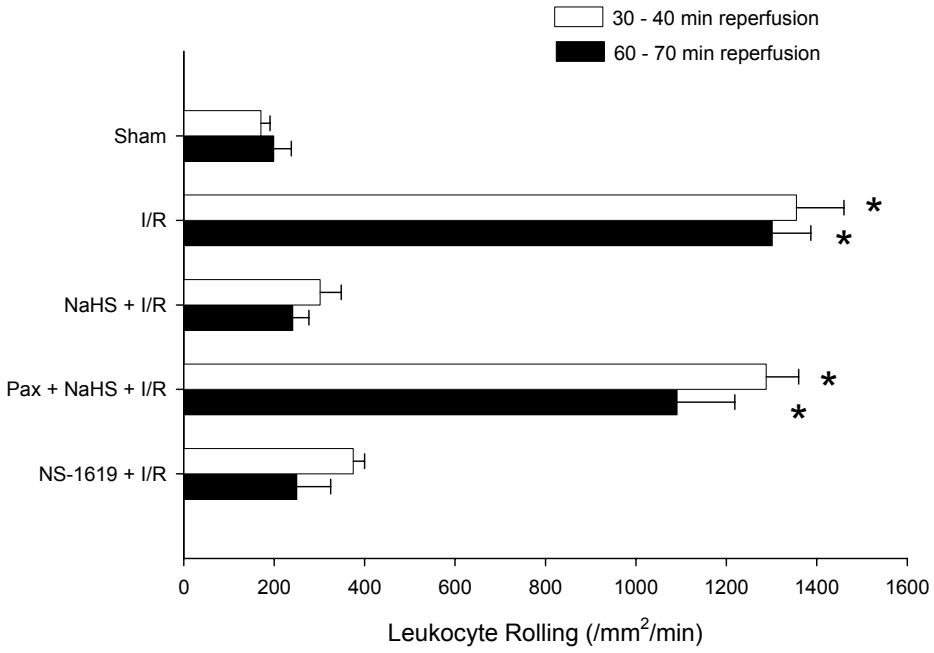

Day 1
24 hrs
Day 2
min
-10
0
-45
0
30
60
Group 1 Sham
Saline
Saline
Group 2 I/R
Ischemia (45min)
Saline
Saline
Group 3 NaHS + I/R
Saline
NaHS
Group 4 Paxilline + NaHS + I/R
Pax
NaHS
Group 5 NS-1619 + I/R
Saline
NS-1619
Administration of drugs
Video Recording (10 min)

Figure 2


30 - 40 min reperfusion
60 - 70 min reperfusion
Sham
I/R
NaHS + I/R
Pax + NaHS + I/R
NS-1619 + I/R
*
*
*
*
0
200
400
600
800
1000
1200
1400
1600
Leukocyte Rolling (/mm2/min)

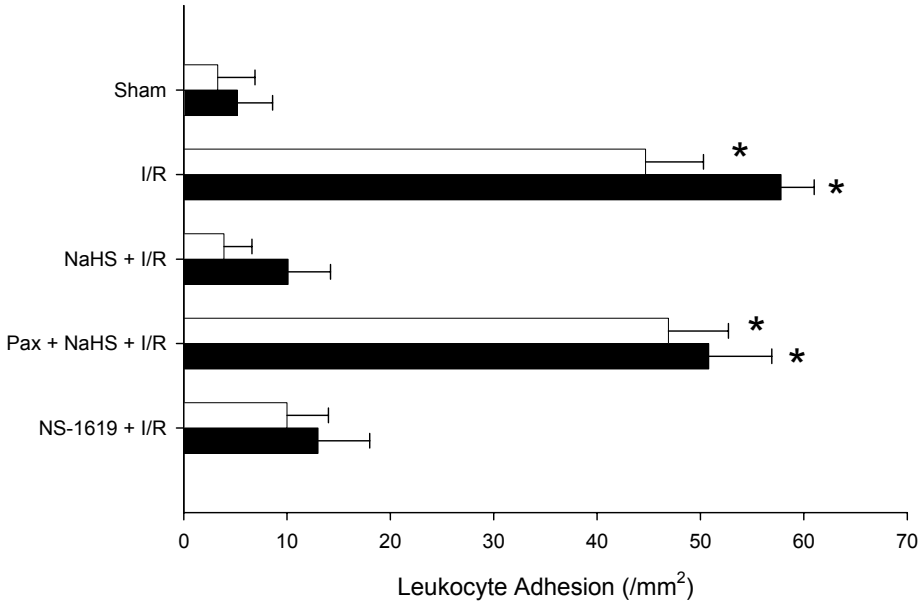
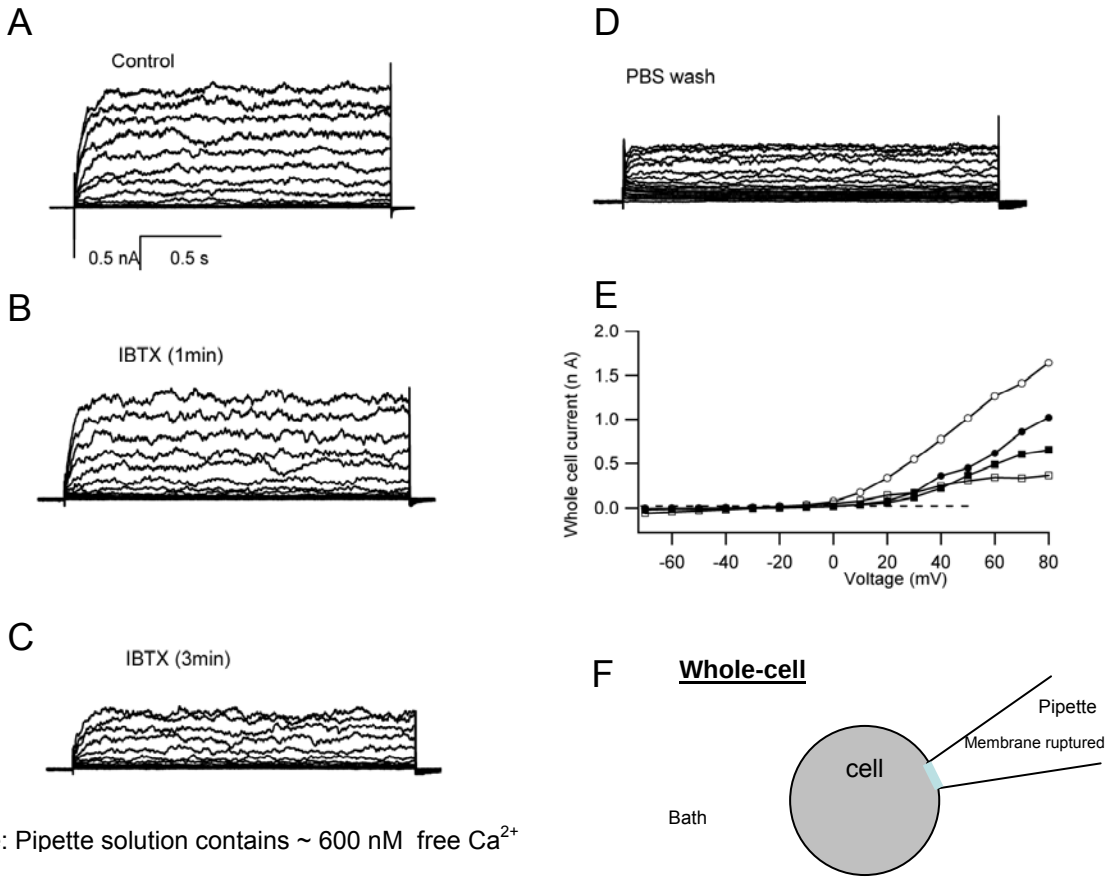

Sham
I/R
NaHS + I/R
Pax + NaHS + I/R
NS-1619 + I/R
*
*
*
*
0
10
20
30
40
50
60
70
Leukocyte Adhesion (/mm2)

Figure 3




Note: Pipette solution contains ~ 600 nM free $Ca^{2+}$

Figure 4

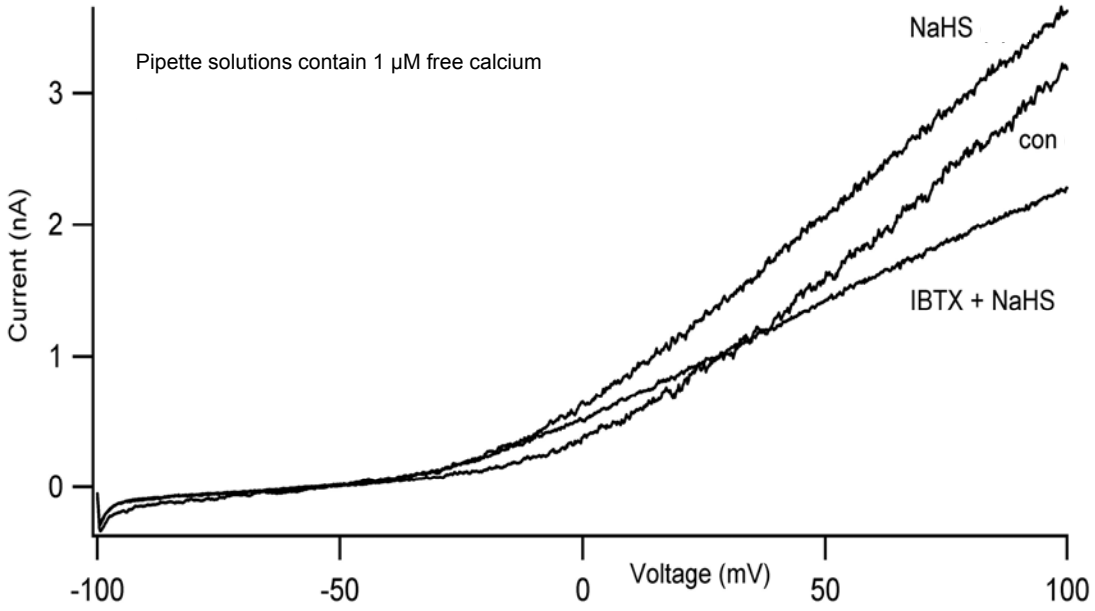

Pipette solutions contain 1 μM free calcium
NaHS
con
IBTX + NaHS
Current (nA)
3
2
1
0
-100
-50
0
50
100
Voltage (mV)

Figure 5

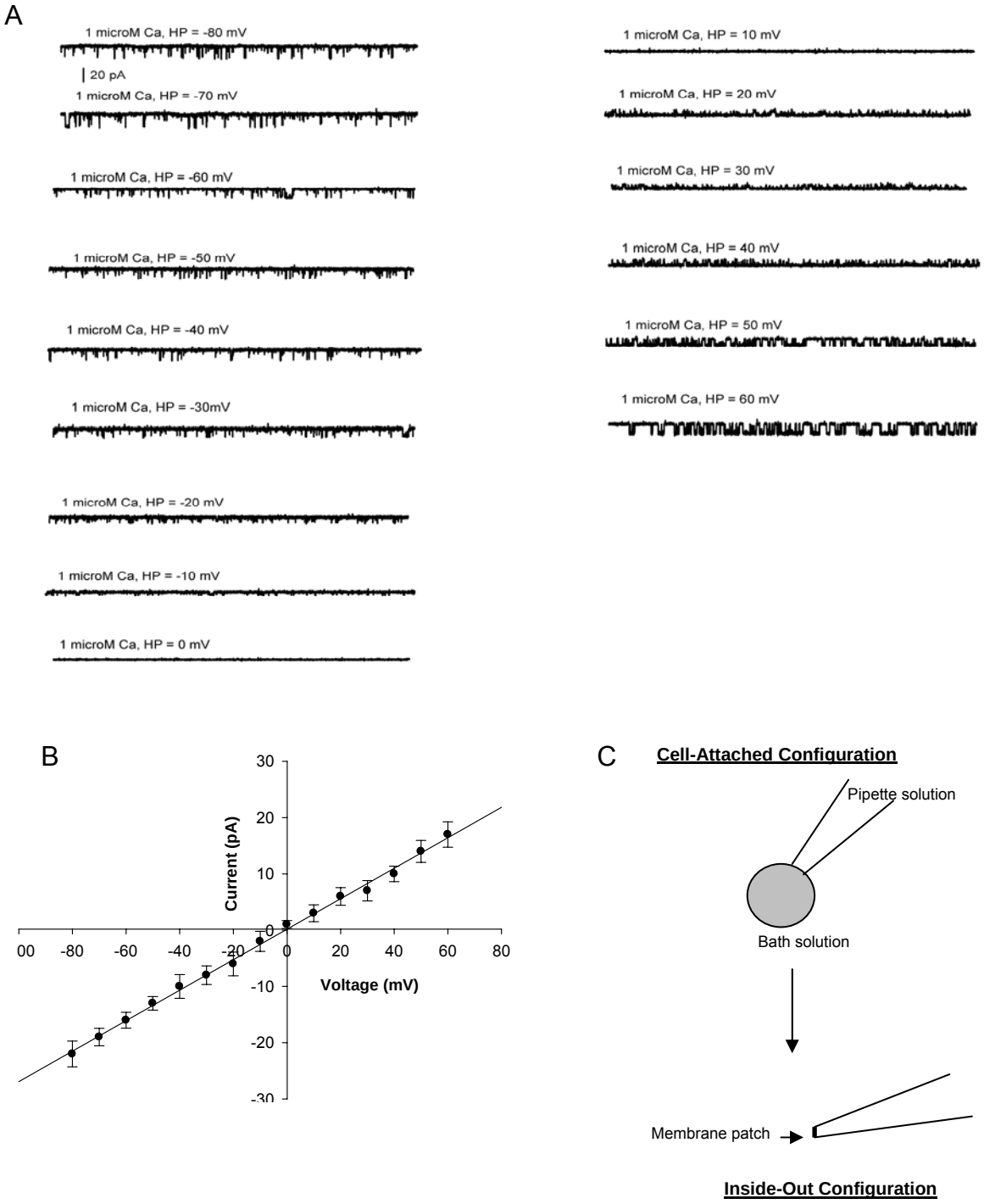
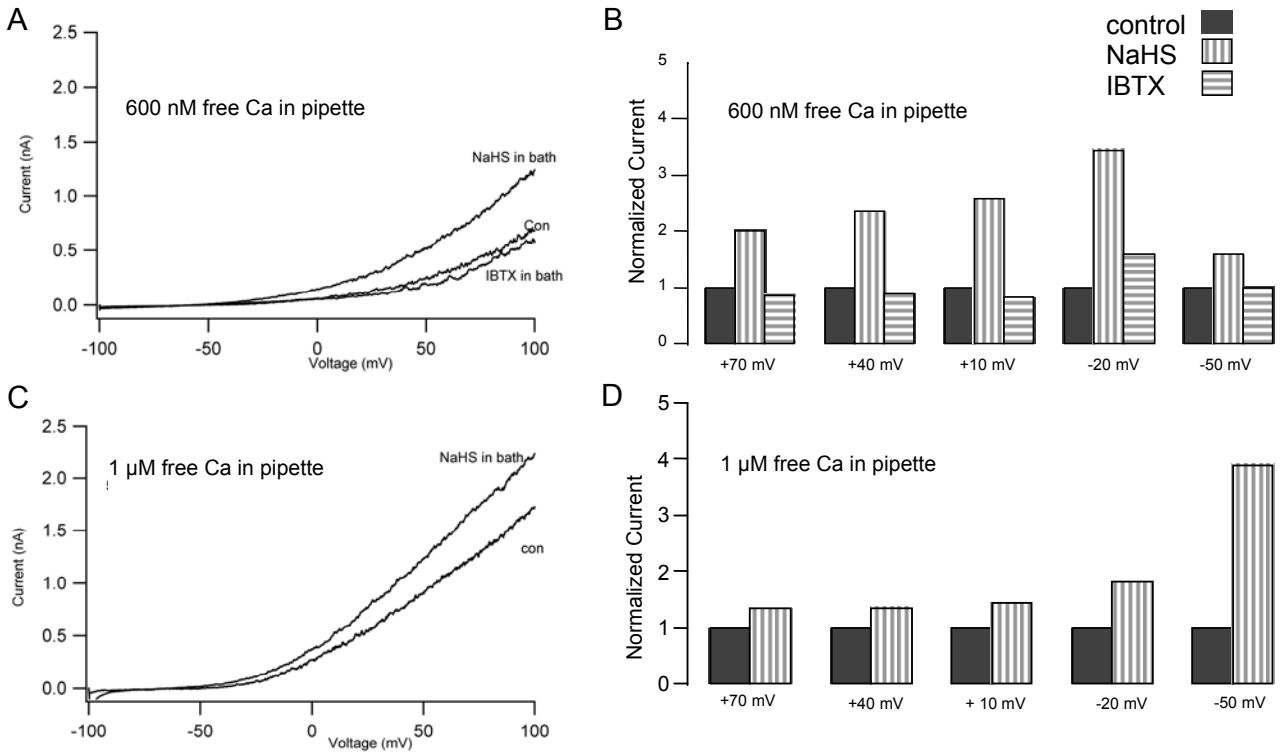

A
1 microM Ca, HP = -80 mV
20 pA
1 microM Ca, HP = -70 mV
1 microM Ca, HP = -60 mV
1 microM Ca, HP = -50 mV
1 microM Ca, HP = -40 mV
1 microM Ca, HP = -30mV
1 microM Ca, HP = -20 mV
1 microM Ca, HP = -10 mV
1 microM Ca, HP = 0 mV
1 microM Ca, HP = 10 mV
1 microM Ca, HP = 20 mV
1 microM Ca, HP = 30 mV
1 microM Ca, HP = 40 mV
1 microM Ca, HP = 50 mV
1 microM Ca, HP = 60 mV
B
Current (pA)
Voltage (mV)
30
20
10
0
-10
-20
-30
00
-80
-60
-40
-20
0
20
40
60
80
C
Cell-Attached Configuration
Pipette solution
Bath solution
Membrane patch
Inside-Out Configuration

Figure 6


A
600 nM free Ca in pipette
NaHS in bath
Con
IBTX in bath
Current (nA)
Voltage (mV)
B
control
NaHS
IBTX
600 nM free Ca in pipette
Normalized Current
+70 mV
+40 mV
+10 mV
-20 mV
-50 mV
C
1 µM free Ca in pipette
NaHS in bath
con
Current (nA)
Voltage (mV)
D
1 µM free Ca in pipette
Normalized Current
+70 mV
+40 mV
+ 10 mV
-20 mV
-50 mV

# ANTECEDENT HYDROGEN SULFIDE ELICITS AN ANTI-INFLAMMATORY PHENOTYPE IN POSTISCHEMIC MURINE SMALL INTESTINE: ROLE OF HEME OXYGENASE-1

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*Running head:* Hydrogen sulfide preconditioning: role of HO-1

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## ABSTRACT

We recently demonstrated that preconditioning with an exogenous hydrogen sulfide donor ($H_2S$) donor (NaHS-PC) 24 h prior to ischemia and reperfusion (I/R) causes postcapillary venules to shift to an anti-inflammatory phenotype in C57Bl/6 wild-type mice such that these vessels fail to support increased postischemic LR and LA. We have also previously shown that this pattern of $H_2S$-induced late-phase preconditioning involves triggers that normally mediate vasorelaxation: eNOS , $K_{ATP}$ and BK channel activation. The objective of the present study was to determine whether heme oxygenase-1 is a mediator of the anti-inflammatory effects noted during I/R in mice preconditioned with NaHS. I/R induced marked increase in LR and LA, effects that were prevented by NaHS-PC. Treatment with the HO inhibitor, tin protoporphyrin IX (SnPPIX), but not the inactive protoporphyrin CuPPIX, just prior to reperfusion prevented the anti-inflammatory effects of antecedent NaHS. We then evaluated the effect of NaHS as a preconditioning stimulus in mice that were genetically deficient in HO-1 (HO-1 -/- in the H129 background with appropriate WT controls). NaHS-PC was ineffective in HO-1-/- mice. Our work indicates that HO-1 serves as an effector of the anti-inflammatory effects of NaHS-PC during I/R 24 hrs later.



**Keywords:** ischemia, reperfusion, leukocyte rolling, leukocyte adhesion, preconditioning

## INTRODUCTION

We recently demonstrated that administration of the hydrogen sulfide donor NaHS 24 hrs prior to ischemia/reperfusion (NaHS preconditioning or NaHS) prevents the increases in postischemic leukocyte rolling and adhesion in murine small intestine (Yusof et al., in press). Although these studies provided evidence that eNOS, p38-MAPK, KATP, and BKCa channel activation play critical roles as triggers for the development of this form of preconditioning, the effector(s) that mediate the anti-inflammatory effects of NaHS-PC during I/R 24 hrs later remain unknown. However, recent work has shown that exogenous administration of $H_2S$ increases heme oxygenase-1 (HO-1) expression (16). Because the expression of HO-1 is preferentially localized to the jejunum and ileum, regions of the intestine that are particularly susceptible to I/R injury, and the reaction products of HO-1-mediated heme degradation exhibit powerful anti-adhesive and antioxidant properties (18), we postulated that this heat shock protein may serve as an essential mediator of NaHS-PC during I/R 24 hrs later, preventing oxidant-induced leukocyte-endothelial cell interactions. Thus, the aim of this study was to evaluate the role of HO-1 as an effector of NaHS-PC. To accomplish this aim, we employed a pharmacologic inhibitor approach directed at reducing HO-1 activity during I/R and evaluated the effectiveness of this $H_2S$ donor in mice that were genetically deficient in HO-1.

## MATERIALS AND METHODS

*Animals:* Wild-type (WT) male C57B/L6J WT mice (6-7 weeks of age), as well as WT H129 (HO-1 +/+) were obtained from the Jackson Laboratories (Bar Harbor, ME). A breeding colony of HO-1 -/- mice in the H129 background were obtained by a generous gift from Dr. William Fay (University of Missouri – Columbia) and bred for HO-1 -/- mice used in the experiments. All mice were maintained on standard mouse chow and used at 8-12 weeks of age. The experimental procedures described herein were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri – Columbia Institutional Animal Care and Use Committee.

*Surgical Procedures and Induction of I/R:* The mice were anesthetized initially with the mixture of ketamine (150 mg/kg body wt, i.p.) and xylazine (7.5 mg/kg body wt, i.p.). The right carotid artery was cannulated and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery catheter. Systemic blood pressure was recorded continuously with a personal computer (Power Macintosh 8600; Apple) equipped with an analog-to-digital converter (MP 100; Biopac Systems). Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE, Molecular Probes, Eugene, OR, USA) was dissolved in DMSO at a stock concentration of 5 mg/ml, divided into 25 µl aliquots, and stored in light-tight containers at -20 °C until further dilution immediately prior to intravenous injection. The left jugular vein was cannulated for administration of CFDA-SE. After these procedures, a midline abdominal incision was performed, the superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (sham) or 45 min. After the ischemic period, the clip was gently

removed and leukocytes were labeled with CFDA-SE by intravenous administration of the fluorochrome solution (250 μg/ml saline) at 20 μl/min for 5 min. During the preparation, storage, and administration of CFDA-SE, care was taken to minimize light exposure. Leukocyte/endothelial cell adhesive interactions were quantified over min 30-40 and 60-70 of reperfusion, as described below.

*Intravital Fluorescence Microscopy:* The mice were positioned on a 20 × 30-cm Plexiglas board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 × 3-cm hole centered in the Plexiglas. The exposed small intestine was superfused with warmed (37 °C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min using a peristaltic pump (Model M312; Gilson). The exteriorized region of the small bowel was covered with BBS-soaked gauze to minimize the tissue dehydration, temperature changes, and the influence of respiratory movements. The superfusate was maintained at 37 ± 0.5 °C by pumping the solution through a heat exchanger warmed by a constant-temperature circulator (Model 1130; VWR). Body temperature of the mouse was maintained between 36.5 and 37.5 °C by use of a thermostatically controlled heat lamp. The board was mounted on the stage of an inverted microscope (Diaphot TMD-EF; Nikon) and the intestinal microcirculation was observed through a 20× objective lens. Intravital fluorescence images of the microcirculation (excitation wavelength, 420-490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics) and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor

(PVM-1953MD; Sony) and recorded on digital video using a digital video recorder (DMR-E50; Panasonic) for off-line quantification of measured variables during playback of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function onto the monitor.

The intravital microscopic measurements described below were obtained over minutes 30-40 and 60-70 of reperfusion or at equivalent time points in the control groups. The intestinal segment was scanned from the oral to aboral section and 10 single, unbranched venules (20-50 μm diameter, 100 μm length) were observed, each for 30 sec. Leukocyte-endothelial cell interactions (the numbers of rolling and firmly adherent leukocytes) were quantified in each of the 10 venules, followed by calculation of the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move or detach from the venular wall for a period equal to or greater than 30 sec. Rolling cells are defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than centerline velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adherent leukocytes were normalized by expressing each as the number of cells per $mm^2$ vessel area.

*Experimental Protocols:* The general design of the experimental protocols for each group in the study is described below.

***Group 1: Sham.*** As a time control for the effects of experimental duration, the mesentery of each mouse in this group (n=6) was superfused with bicarbonate-buffered saline. The superior mesenteric artery was exposed but not subjected to occlusion, with leukocyte-endothelial cell adhesive interactions quantified at time points comparable to

those described for mice subjected to 45 min of intestinal ischemia followed by 70 min reperfusion (Group 2, below).

***Group 2: I/R alone.*** Mice in this group (n=6) were treated as described for Group 1 above except that I/R was induced by occlusion of the superior mesenteric artery for 45 min followed by reperfusion for 70 min. Leukocyte rolling and adhesion were quantified during min 30-40 and 60-70 of reperfusion.

***Group 3: NaHS + I/R.*** In order to determine whether $H_2S$ can act as a preconditioning stimulus and prevent I/R-induced LR and LA, a solution of NaHS was used as a $H_2S$ donor. NaHS (Sigma Chemical, St. Louis, MO) was weighed and diluted in saline at a concentration of 1.4 mM). Mice in this group (n=6) were treated as described for Group 2 except that NaHS (1.4 mM x (weight g/10) mL injected) was administered 24 hrs prior to I/R.

***Group 4: NaHS + SnPPIX + I/R.*** To explore the mechanism underlying the development of the anti-inflammatory phenotype induced by late phase $H_2S$-PC, we investigated the role of HO-1 as an effector in NaHS-PC. Initial investigation was carried out by treating mice with the HO-1 inhibitor, tin protoporphyrin (SnPPIX) (15 mmol/L, 0.1mL/30g mouse). Mice in this group (n=6) were treated as described for Group 3 except that SnPPIX was administered 60 minutes prior to reperfusion.

***Group 5: NaHS + CuPPIX + I/R.*** As a negative control, and inactive protoporphyrin, (copper protoporphyrin (CuPPIX, 15 mmol/L, 0.1mL/30g mouse)) that exhibits no inhibitory activity toward HO, was administered as described for SnPPIX in Group 4 (n=6).

***Group 6: Hemin + I/R***. In order to determine whether HO-1 induction, on it's own, would precondition the post-capillary venules against leukocyte rolling and adhesion, we administered hemin (Sigma Chemical, St. Louis, MO), in lieu of NaHS, 24 hrs prior to I/R.

Mice genetically deficient in HO-1 (HO-1-/-) and their wild-type littermates were used in the remaining groups to provide molecular genetic evidence for a role for this particular heme oxygenase isoform in NaHS-PC, as well as to provide additional support for the notion the heme oxygenase plays a critical role in the anti-inflammatory effects of antecedent treatment with the $H_2S$ donor.

**Groups 7-9: *Sham (HO-1 +/+***), ***I/R (HO-1 +/+), NaHS + I/R (HO-1 +/+).*** The protocols outlined for Groups 1-3, respectively, were repeated in WT littermates of the HO-1 knockout animals, because the knockouts were developed on an H129 mouse background. This allowed us to compare the effects of I/R and the effectiveness of NaHS as a preconditioniong stimulus in this strain vs the C57BL/6J mouse used in our previous studies and in Groups 1-3 above (n=5 for all groups). The data are similar to that found in C57BL/6J background, and are not presented here.

***Groups 10-12: Sham (HO-1 -/-*** ), ***I/R (HO-1 -/-), NaHS + I/R (HO-1 -/-).*** To evaluate the effect of genetic HO-1 ablation on the effectiveness of NaHS as a preconditioning stimulus, the studies outlined for Groups 1-3, respectively, above were again repeated in HO-1 knockout mice with H129 background (n=4 in all groups). The data are presented in Figure 3.

*Statistical Analysis:* The data were analyzed with standard statistical analysis, i.e., ANOVA with Sheffe's (post hoc) test for multiple comparisons. All values are expressed as means ± SEM. Statistical significance was defined at $P < 0.05$.

## RESULTS

Figure 2 illustrates the average numbers of rolling (Upper Panel) and adherent (Lower Panel) leukocytes in postcapillary venules of the murine small intestine of C57BL/6J wild-type mice exposed to I/R alone (I/R, Group 2) or $H_2S$ donor 24 hrs prior to I/R (NaHS + I/R, Group 3) relative to non-ischemic controls (Sham, Group 1). I/R induced marked increases in the numbers of rolling and adherent leukocytes after 30 and 60 min of reperfusion, proadhesive effects that were abolished by preconditioning with the $H_2S$ donor, NaHS.

To initially investigate the our hypothesis that heme oxygenase serves as an essential mediator of the anti-adhesive effects of NaHS-PC, postischemic LR and LA were quantified in C57BL/6J WT mice treated with the heme oxygenase inhibitor SnPPIX and the negative-control protoporphyrin CuPPIX prior to I/R 24 hrs after $H_2S$ donor administration (Figure 2). SnPPIX, but not CuPPIX, completely abrogated the anti-adhesive effects of NaHS-PC when administered prior to I/R. To further explore this question, we preconditioned animals by treatment with hemin 24 hrs prior to I/R. Antecedent treatment with this heme oxygenase inducer was as effective as NaHS-PC in preventing the increases in leukocyte rolling and adhesion induced by I/R 24 hrs after preconditioning with hemin. These results suggest that heme oxygenase may serve as an important mediator of NaHS-PC during I/R.

The data depicted in Figure 3 illustrate the effects of I/R alone and NaHS-PC + I/R in HO-1 knockout animals and their wild-type littermates. Baseline and postischemic leukocyte rolling were similar in both strains of mice and were not different than their respective values noted in C57/BL6 mice. Similarly, NaHS-PC was as effective in

limiting I/R-induced leukocyte rolling and adhesion in H129 wild-type mice as compared to C57BL/6J mice. However, antecedent treatment with NaHS was completely ineffective as a preconditioning stimulus in mice that were genetically-deficient in HO-1. The latter observation provides a third line of evidence supporting a role for this heat shock protein in the anti-adhesive phenotype that develops in response to $H_2S$ preconditioning. Moreover, our data indicate that the HO-1 isoform plays a dominant role in NaHS-PC.

## DISCUSSION

Our previous work demonstrated roles for NaHS-dependent eNOS, p38-MAPK, $K_{ATP}$, and $BK_{Ca}$ channel activation as critical triggering elements that inaugurate or trigger entrance into a preconditioned anti-inflammatory state (Yusof et al., AJP manuscripts under review). However, the identity of the effector(s) or mediator(s) of the anti-adhesive effects of NaHS-PC were largely unexplored. The observation that $H_2S$ upregulates the expression of HO-1 by Qingyou et al. (16) was of particular interest to us because the catalytic products of HO-1-mediated heme metabolism exhibit powerful anti-oxidant and anti-adhesive properties. Because I/R induces oxidant-dependent leukocyte rolling and adhesion (24), we hypothesized that NaHS-PC may be mediated by upregulated expression of this heat shock protein.

Heme oxygenase (HO) is a ubiquitously expressed protein that catalyzes the oxidative degradation of protoheme IX into equimolar quantities of biliverdin, divalent iron, and carbon monoxide (CO). Biliverdin is further metabolized to bilirubin, a powerful endogenous antioxidant, by the action of biliverdin reductase. Three isoforms of the enzyme, HO-1, HO-2, and HO-3, have been described (14). HO-3 appears to exhibit lower activity and is less well characterized than HO-1 and HO-2, and may be a splice variant of HO-2. HO-2 is a constitutively expressed and non-inducible gene product. On the other hand, HO-1 is an inducible enzyme which is regarded as a heat shock protein (HSP32) in animal models.

We hypothesized that heme oxygenase might serve as an effector of NaHS-PC for the following reasons. First, heme oxygenase activity is exquisitely sensitive to upregulation by NO donors (9, 18) and NO appears to play an important role in initiating

the effects of NaHS-PC (Yusof et al., AJP, manuscript under review). Second, induction of HO-1 suppresses P-selectin expression and leukocyte adhesion induced by hydrogen peroxide or ischemia/reperfusion in the small intestine (23), inflammatory processes which are also prevented by NaHS-PC. Third, hemin-induced HO-1 expression exerts infarct-sparing effects in the setting of myocardial I/R (3, 10), whereas HO-1 knockout animals do not develop a preconditioned phenotype in response to brief I/R (2, 13). Moreover, HO-1 activity appears to be particularly rich in postcapillary venules of the small intestine (7). Finally, and perhaps most importantly, the reaction products of HO-1-catalyzed hemin degradation exert powerful anti-adhesive and antioxidant effects (6, 15, 20).

To begin to address this postulate, we evaluated the effects of $H_2S$ donor treatment on HO-1 in the small intestine and vascular tissue. Our finding that NaHS increased HO-1 expression in these tissues confirmed and extended the earlier work of Qingyou et al (16) who demonstrated a similar effect in the lung. We then turned our attention to the possibility that treatment with a non-isoform selective pharmacologic inhibitor of heme oxygenase would prevent the anti-inflammatory effects of NaHS-PC when administered during I/R 24 hrs later. Pharmacologic inhibition of HO with tin protoporphyrin (SnPPIX), but not the inactive protoporphyrin, copper protoporphyrin (CuPPIX), completely prevented the anti-adhesive actions of antecedent NaHS. We also demonstrated that preconditioning with an agent known to upregulate the expression and activity of HO-1 (hemin), 24 hrs prior to I/R was as effective as antecedent NaHS in preventing postischemic leukocyte rolling and adhesion.

While the aforementioned studies provide strong support for the notion that heme oxygenase serves as an effector of NaHS-PC, the pharmacologic inhibitor studies fail to identify the heme oxygenase isoform responsible for these anti-inflammtory actions. In addition, the studies rely on the specificity of SnPPIX as a heme oxygenase inhibitor. Indeed, there is evidence suggesting that this agent also inhibits inducible NOS, an enzymatic source of NO, a gaseous monoxide that not only exerts anti-adhesive effects but has also been implicated in other forms of preconditioning. In light of these issues, we evaluated the effectiveness of NaHS as preconditioning stimulus in HO-1 knockout mice and demonstrated that the anti-adhesive effects antecedent treatment with this $H_2S$ were completely absent. Taken together, our studies provide compelling support for the notion that HO-1 is a critically important molecular end-effector of the anti-adhesive phenotype elicited by NaHS-PC.

A growing body of evidence indicates that $H_2S$ exerts a variety of effects that may limit I/R-induced injury and inflammation. For example, $H_2S$ produces vasorelaxation by activating $K_{ATP}$ channels (1, 4, 8, 11, 22, 26, 27) and can enhance the vasodilatory effects of NO (8, 25), actions that may improve tissue perfusion in I/R. In addition, this gaseous signaling molecule reduces mitochondrial respiration, which may conserve ATP levels in ischemic tissues . $H_2S$ also exerts antioxidant effects and limits oxidative stress by virtue of its actions to raise intracellular glutathione levels by enhancing the activity of γ-glutamylcysteine synthetase and upregulating cysteine transport (12). Additionally, $H_2S$ enhances the ability of superoxide dismutase to scavenge superoxide (19). Furthermore, $H_2S$ reduces apoptosis induced by inhibition of caspase-3 cleavage and p38 MAPK phosphorylation (17) which may contribute to potential infarct-sparing

effects in I/R. Moreover, inhibition of endogenous $H_2S$ synthesis reduces leukocyte rolling velocity and increases leukocyte adherence to mesenteric postcapillary venules under baseline conditions while NaHS treatment prevent leukocyte-endothelial cell adhesive interactions following exposure to TNF$\alpha$ (5). The former results indicate that basal $H_2S$ production serves as an endogenous modulator of leukocyte/endothelial cell adhesive interactions while the latter observation indicates that $H_2S$ donors may be effective in preventing adhesive responses induced by proinflammatory mediators. Our results indicate that in addition to the potential use of $H_2S$ donors such as NaHS or interventions, as yet undiscovered, that upregulate of CBS and CSE during I/R, antecedent treatment (ie, preconditioning) with NaHS induces the development of an anti-adheseive phenotype such that postcapillary venules are able to resist the pro-inflammatory effects of I/R 24 hrs later. Thus, NaHS-PC holds clinical promise as a prophylactic treatment for the reduction of the inflammatory component of I/R when tissue ischemia can be anticipated (eg, cardiopulmonary bypass, transplantation, operation in a bloodless field) and may also be useful in reducing the likelihood of adverse cardiovascular effects that arise unexpectedly (eg, mesenteric occlusion, compartment syndrome, myocardial infarction, stroke).

In summary, the results of our study provide four lines of evidence implicating HO-1 as an important mediator or effector of the anti-adhesive effects of antecedent NaHS treatment. First, we demonstrated that this $H_2S$ donor upregulates the expression of HO-1 in the small intestine and vascular tissues. Second, inhibition of heme oxygenase activity during I/R completely abrogated the ability of antecedent NaHS to prevent postischemic leukocyte rolling and adhesion. Third, the anti-adhesive

effects induced by NaHS-PC could be mimicked by preconditioning with the HO-1 induced, hemin. Finally, the anti-adhesive effects of NaHS-PC were completely absent in mice that were genetically-deficient in HO-1.

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## FIGURE LEGENDS

**Figure 1.** ***Experimental Protocol:*** Illustration of the experimental protocols assigned to each group. The numbers along the top of the diagram in minutes refer to the time line of the protocol on Day 1, 24 hours prior to ischemia and reperfusion (I/R), and Day 2, the day of I/R. Hatched bars indicate digital video recording (10 min). Solid black bars indicate the 45 min period of ischemia during which the superior mesenteric artery had no blood flow. Triangles indicate administration of drug. See text for further details.

**Figure 2.** ***Effects of pharmacologic HO-1 inhibition in C57BL/6J mice on the preconditioning effects of NaHS.*** Effects of ischemia/reperfusion alone (I/R), I/R following pretreatment with a $H_2S$ donor (NaHS + I/R), or HO-1 inhibition just prior to treatment with $H_2S$ donor (SnPPIX + NaHS +I/R), and HO-1 induction in lieu of NaHS (Hemin + I/R) on postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion. * indicates mean values that were statistically different from the NaHS + IR group at $p<0.05$.

**Figure 3.** ***Effects of genetic deletion of HO-1 gene in H129 mice on the preconditioning effects of NaHS.*** Effects of ischemia/reperfusion alone (I/R), I/R following pretreatment with a $H_2S$ donor (NaHS + I/R) on postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion. * indicates mean values that were statistically different from the Sham group at $p<0.05$.

Figure 1

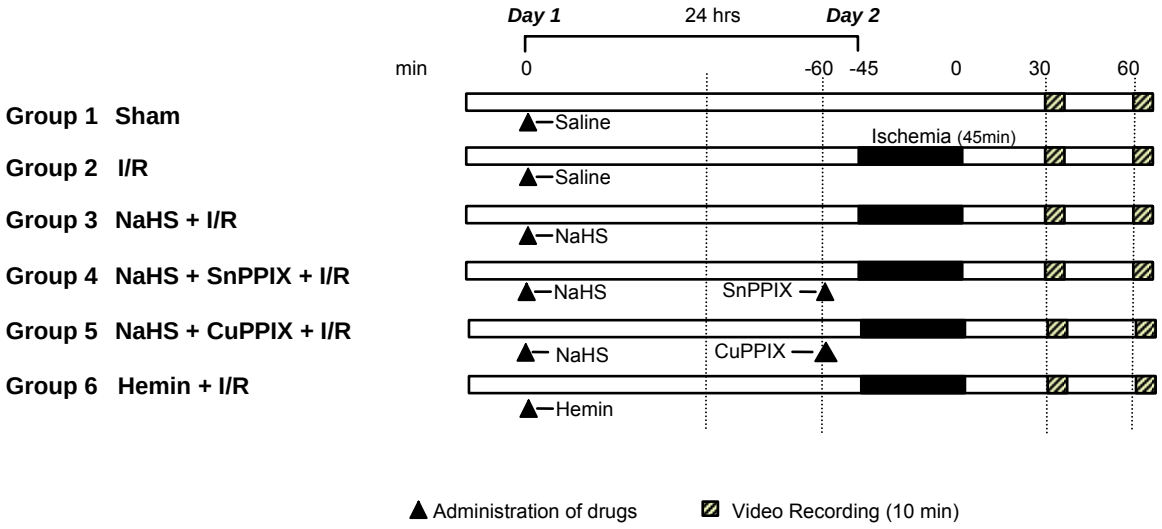
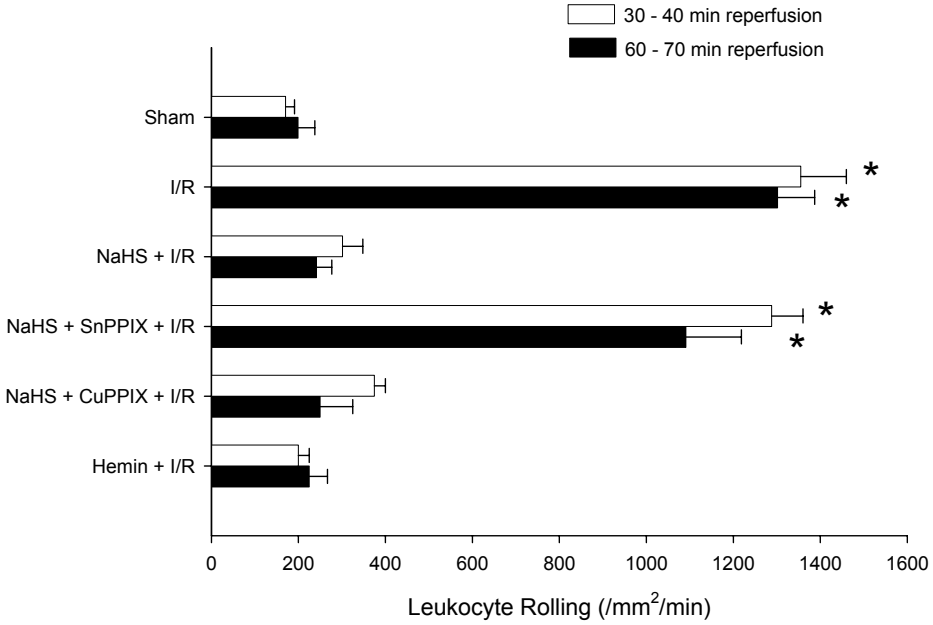

Day 1
24 hrs
Day 2
min
0
-60
-45
0
30
60
Group 1 Sham
Saline
Group 2 I/R
Ischemia (45min)
Saline
Group 3 NaHS + I/R
NaHS
Group 4 NaHS + SnPPIX + I/R
NaHS
SnPPIX
Group 5 NaHS + CuPPIX + I/R
NaHS
CuPPIX
Group 6 Hemin + I/R
Hemin
Administration of drugs
Video Recording (10 min)

Figure 2


30 - 40 min reperfusion
60 - 70 min reperfusion
Sham
I/R
NaHS + I/R
NaHS + SnPPIX + I/R
NaHS + CuPPIX + I/R
Hemin + I/R
*
*
*
*
0
200
400
600
800
1000
1200
1400
1600
Leukocyte Rolling (/mm2/min)

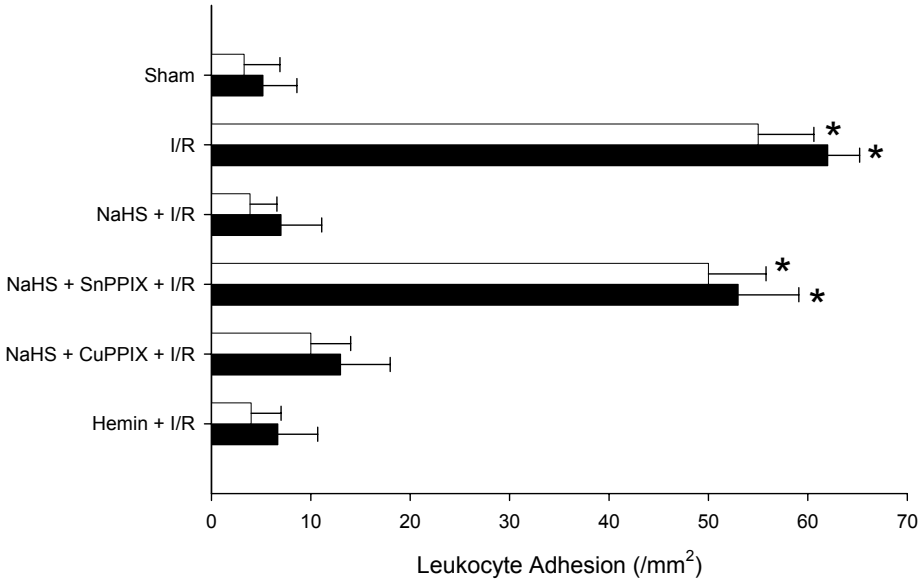
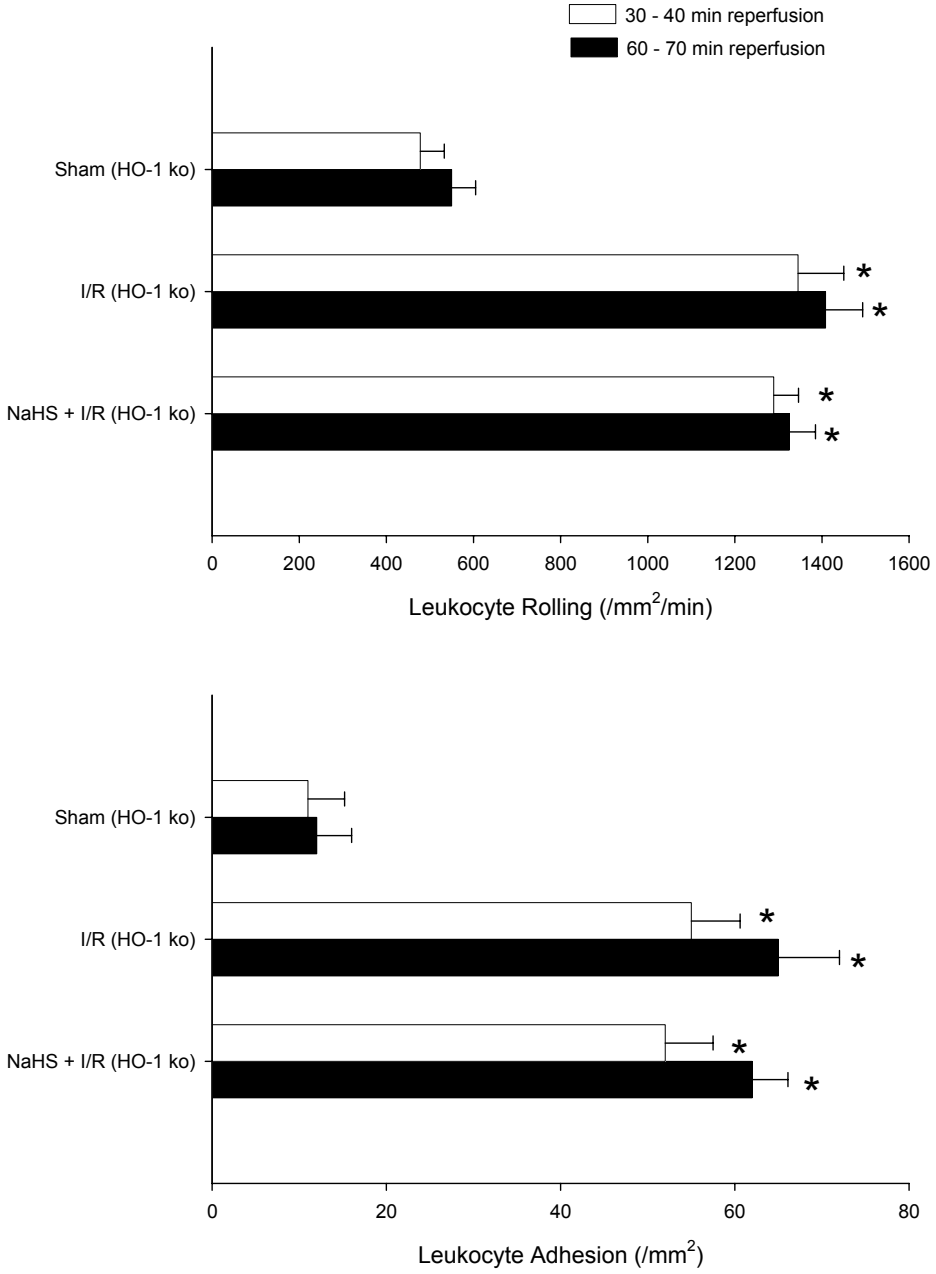

Sham
I/R
NaHS + I/R
NaHS + SnPPIX + I/R
NaHS + CuPPIX + I/R
Hemin + I/R
*
*
*
*
0
10
20
30
40
50
60
70
Leukocyte Adhesion (/mm2)

Figure 3


30 - 40 min reperfusion
60 - 70 min reperfusion
Sham (HO-1 ko)
I/R (HO-1 ko)
NaHS + I/R (HO-1 ko)
*
0
200
400
600
800
1000
1200
1400
1600
Leukocyte Rolling (/mm2/min)
Sham (HO-1 ko)
I/R (HO-1 ko)
NaHS + I/R (HO-1 ko)
*
0
20
40
60
80
Leukocyte Adhesion (/mm2)

# ANGIOTENSIN II MEDIATES POSTISCHEMIC LEUKOCYTE-ENDOTHELIAL INTERACTIONS: ROLE OF CALCITONIN GENE-RELATED PEPTIDE

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*Running head:* Angiotensin II, CGRP, and postischemic inflammation

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## ABSTRACT

Vascular inflammation and enhanced production of angiotensin II (Ang II) are involved in the pathogenesis of hypertension and diabetes, disease states that predispose the afflicted individuals to ischemic disorders. Based on these observations, we postulated that Ang II may play a role promoting leukocyte rolling (LR) and adhesion (LA) in postcapillary venules after exposure of the small intestine to ischemia/reperfusion (I/R). Using an intravital microscopic approach in C57BL/6J mice, we showed that Ang II $AT_1$ or $AT_2$ receptor antagonism (with valsartan or PD123,319, respectively), inhibition of angiotensin converting enzyme (ACE) with captopril, or CGRP receptor blockade (CGRP8-37) prevented postischemic LR but did not influence I/R-induced LA. However, both postischemic LR and LA were largely abolished by concomitant $AT_1$ and $AT_2$ receptor blockade or chymase inhibition (with Y-40079). Additionally, exogenously administered Ang II increased LR and LA, effects that were attenuated by pretreatment with a CGRP receptor antagonist or an NADPH oxidase inhibitor (apocynin). Our work suggests that Ang II, formed by the enzymatic activity of ACE and chymase, plays an important role in inducing postischemic LR and LA, effects that involve engagement of both $AT_1$ or $AT_2$ receptors and may be mediated by CGRP and NADPH oxidase.



**Keywords:** ischemia, reperfusion, leukocyte rolling, leukocyte adhesion, angiotensin converting enzyme, chymase, angiotensin $AT_1$ and $AT_2$ receptors, NADPH oxidase

## INTRODUCTION

Angiotensin II (Ang II) is the main effector of the renin-angiotensin system, acting as a potent vasoconstrictor and key regulator of blood pressure and electrolyte homeostasis (6). An emerging body of evidence indicates that in addition to these well-known actions, Ang II also stimulates a wide variety of pro-inflammatory responses including increased leukocyte rolling and adhesion, production of oxidative stress, and induction of CXC chemokine expression (27). Ang II is formed by the enzymatic action of angiotensin converting enzyme (ACE) and mast cell-derived chymase and mediates its effects by binding to specific cell surface receptors (9, 13). In the cardiovascular system, the main angiotensin receptor subtypes include type I ($AT_1$) and type II ($AT_2$) receptors (8). In contrast to the well-established physiological role of $AT_1$ receptors in the cardiovascular effects of Ang II, the significance of $AT_2$ receptor activation is not well characterized. However, exogenous Ang II has been shown to increase leukocyte rolling and adhesion via $AT_1$ and $AT_2$ receptor-mediated P-selectin expression (25). More recent work indicates that the increase in leukocyte-endothelial cell adhesive interactions induced by intestinal ischemia and reperfusion (I/R) can be prevented by treatment with an $AT_1$ receptor antagonist (24).

While the aforementioned studies clearly establish a role for Ang II in postischemic leukosequestration in intestinal tissues, the source of this inflammatory octapeptide (ACE vs chymase) and the relative contribution of $AT_1$ vs $AT_2$ receptors to increased leukocyte rolling and adhesion in the small bowel are undefined. In addition, the downstream signaling mechanisms underlying postischemic Ang II-dependent leukocyte rolling and adherence are unclear. However, it is well-established that Ang II increases

vascular and neutrophilic superoxide production by an NADPH oxidase-dependent mechanism (11, 34). In addition, recent work indicates that Ang II increases circulating levels of calcitonin gene-related peptide (CGRP) (20, 26, 35). Since I/R-induced leukocyte adhesion occurs by an oxidant-dependent mechanism and because CGRP is a proadhesive neuropeptide that plays an important role in neurogenic inflammation, we hypothesized that Ang II derived from mast cell chymase induces inflammation in postischemic venules of the mouse small intestine via an $AT_1$ and $AT_2$ receptor-dependent mechanism that involves CGRP and NADPH oxidase.

## MATERIALS AND METHODS

*Animals:* Wild-type male C57BL/6J mice (6-7 weeks of age) were obtained from the Jackson Laboratories (Bar Harbor, ME). All mice were maintained on standard mouse chow and used at 8-12 weeks of age. The experimental procedures described herein were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri – Columbia Institutional Animal Care and Use Committee.

*Surgical Procedures and Induction of I/R:* The mice were anesthetized initially with the mixture of ketamine (150 mg/kg body wt, i.p.) and xylazine (7.5 mg/kg body wt, i.p.). The right carotid artery was cannulated and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery catheter. Systemic blood pressure was recorded continuously with a personal computer (Power Macintosh 8600; Apple) equipped with an analog-to-digital converter (MP 100; Biopac Systems). Carboxyfluorescein diacetate, succinimidyl ester (CFDASE, Molecular Probes, Eugene, OR, USA) was dissolved in DMSO at a stock concentration of 5 mg/ml, divided into 25 µl aliquots, and stored in light-tight containers at -20 °C until further dilution immediately prior to intravenous injection. The left jugular vein was cannulated for administration of CFDASE. After these procedures, a midline abdominal incision was performed, the superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (sham) or 45 min. After the ischemic period, the clip was gently removed and leukocytes were labeled with CFDASE by intravenous administration of the fluorochrome solution (250 µg/ml saline) at 20 µl/min for 5 min. During the preparation, storage, and administration of CFDASE, care was taken to minimize light exposure.

Leukocyte/endothelial cell adhesive interactions were quantitated over min 30-40 and 60-70 of reperfusion.

*Intravital Fluorescence Microscopy:* The mice were positioned on a 20 × 30-cm Plexiglas board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 × 3-cm hole centered in the Plexiglas. The exposed small intestine was superfused with warmed (37 °C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min using a peristaltic pump (Model M312; Gilson). The exteriorized region of the small bowel was covered with BBS-soaked gauze to minimize the tissue dehydration, temperature changes, and the influence of respiratory movements. The superfusate was maintained at 37 ± 0.5 °C by pumping the solution through a heat exchanger warmed by a constant-temperature circulator (Model 1130; VWR). Body temperature of the mouse was maintained between 36.5 and 37.5 °C by use of a thermostatically controlled heat lamp. The board was mounted on the stage of an inverted microscope (Diaphot TMD-EF; Nikon) and the intestinal microcirculation was observed through a 20× objective lens. Intravital fluorescence images of the microcirculation (excitation wavelength, 420-490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics) and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD; Sony) and recorded on digital video using a digital video recorder (DMR-E50; Panasonic) for off-line quantification of measured variables during playback

of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function onto the monitor.

The intravital microscopic measurements described below were obtained over minutes 30-40 and 60-70 of reperfusion or at equivalent time points in the control groups. The intestinal segment was scanned from the oral to aboral section and 10 single, unbranched venules (20-50 μm diameter, 100 μm length) were observed, each for 30 sec. Leukocyte-endothelial cell interactions (the numbers of rolling and firmly adherent leukocytes) were quantified in each of the 10 venules, followed by calculation of the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move or detach from the venular wall for a period equal to or greater than 30 sec. Rolling cells are defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than centerline velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adherent leukocytes were normalized by expressing each as the number of cells per $mm^2$ vessel area.

*Experimental Protocols:* The general design of the experimental protocols for each group in the study is described below. Drug doses were selected based on reports in the literature (4, 25, 27).

***Group 1: Sham.*** As a time control for the effects of experimental duration, the mesentery of each mouse in this group (n=6) was superfused with bicarbonate-buffered saline, which was also used as the route of administration for the pharmacologic agents used in this study in Groups 3-9 outlined below. The superior mesenteric artery was exposed but not subjected to occlusion, with leukocyte-endothelial cell adhesive

interactions quantified at time points comparable to those described for mice subjected to 45 min of intestinal ischemia followed by 70 min reperfusion (Group 2, below).

***Group 2: I/R alone.*** Mice in this group (n=6) were treated as described for Group 1 above except that I/R was induced by occlusion of the superior mesenteric artery for 45 min followed by reperfusion for 70 min. Leukocyte rolling and adhesion were quantified during min 30-40 and 60-70 of reperfusion.

***Group 3: I/R + Valsartan.*** To determine whether $AT_1$ receptor blockade would prevent I/R-induced leukocyte rolling and adhesion, mice in this group (n=6) were treated as described for Group 2 except that Valsartan (Novartis, East Hanover, NJ, 10 mM) was added to the superfusate 10 min prior to reperfusion.

***Group 4: I/R + PD123,319.*** To determine the effects of $AT_2$ receptor blockade on I/R-induced leukocyte rolling and adhesion, mice in this group (n=6) were treated as described for Group 2 except that PD123,319 (Sigma Aldrich, St. Louis, MO,10 mM) was added to the superfusate 10 min prior to reperfusion.

***Group 5: I/R + Valsartan + PD123,319.*** The effects of combined $AT_1$ and $AT_2$ receptor blockade on I/R-induced leukocyte rolling and adhesion were examined in this group (n=6) by superfusing the mesentery with both Valsartan (10 mM) and PD123,319 (10 mM) beginning 10 min prior to reperfusion.

***Group 6: I/R + Captopril.*** To determine the role of angiotensin concerting enzyme (ACE) in I/R-induced leukocyte rolling and adhesion, mice in this group (n=6) were treated as described for Group 2 except that Captopril (Sigma Aldrich, St. Louis, MO 10 mM) was added to the superfusate 10 min prior to reperfusion.

***Group 7: I/R + Y-40079.*** To evaluate the role of chymase in I/R-induced leukocyte rolling and adhesion, mice in this group (n=6) were treated as described for Group 2 except that Y-40079 (a generous gift from Mitsubishi Pharma, 100 μM) (2) was added to the superfusate 10 min prior to reperfusion.

***Group 8: I/R + CGRP8-37.*** To determine the role of CGRP in I/R-induced leukocyte rolling and adhesion, mice in this group (n=6) were treated as described for Group 2 except that the CGRP receptor antagonist, CGRP8-37, (Sigma Aldrich, St. Louis, MO 10 mM) was added to the superfusate 10 min prior to reperfusion.

***Group 9: I/R + Apocynin.*** The role of NADPH oxidase in postischemic leukocyte rolling and adhesion was interrogated by treating mice in this group (n=3) as described for Group 2 except that apocynin (7.2 mM) was added to the superfusate 10 min prior to reperfusion.

To further explore the mechanisms underlying I/R-induced, Ang II-mediated leukocyte rolling and adhesion, mice were treated with exogenous Ang II (100 nM, Sigma Chemical Co, St Louis) by intraperitoneal injection 2 hrs prior to assessment of LR and LA, in the absence (***Group 10: Ang II,*** (n=6)) and presence of CGRP receptor blockade (***Group 11: Ang II + CGRP8-37,*** (n=6)) or NADPH oxidase (***Group 12: Ang II + apocynin,*** (n=3)) treatment. CGRP8-37 and apocynin were administered via the superfusate, as described for Groups 8 and 9, respectively.

*Statistical Analysis:* The data were analyzed with standard statistical analysis, i.e., ANOVA with Fisher's (post hoc) test for multiple comparisons. All values are expressed as means ± SEM. Statistical significance was defined at $P < 0.05$.

## RESULTS

Figure 1 illustrates the average numbers of rolling (Upper Panel) and adherent (Lower Panel) leukocytes in postcapillary venules of the murine small intestine exposed to I/R alone (I/R, Group 2) or I/R coincident with $AT_1$ receptor blockade (I/R + Valsartan, Group 3) or $AT_2$ receptor blockade (I/R + PD123,319, Group 4) alone, or combined $AT_1$ plus $AT_2$ receptor blockade (I/R + Valsartan + PD123,319, Group 5) relative to non-ischemic controls (Sham, Group 1). I/R induced marked increases in the numbers of rolling and adherent leukocytes after 30 and 60 min of reperfusion. It is important to note that postischemic leukocyte rolling and adhesion appeared to be exclusively confined to postcapillary venules because leukocyte/endothelial cell adhesive interactions were not observed in arterioles in any experiment. The postischemic increase in LR was abolished by $AT_1$ receptor or $AT_2$ receptor blockade alone or coincident administration of $AT_1$ plus $AT_2$ receptor antagonists. However, I/R-induced LA was not affected by treatment with either an $AT_1$ or $AT_2$ receptor antagonist alone, but was largely abolished by concomitant $AT_1$ plus $AT_2$ receptor blockade. These results indicate that I/R-induced leukosequestration occurs by an Ang II-dependent mechanism.

To determine the source of Ang II that mediates postischemic leukocyte rolling and adhesion, we evaluated the effect of mesenteric superfusion with inhibitors of ACE (I/R + Captopril, Group 6) or chymase (I/R + Y-40079, Group 7). Like $AT_1$ or $AT_2$ receptor blockade alone, ACE inhibition abrogated I/R-induced LR (Figure 2, Upper Panel) but did not prevent postischemic LA (Figure 2, Lower Panel). However, chymase inhibition effectively reduced both LR and LA after I/R (Figure 2).

Figure 3 illustrates the effect of CGRP receptor blockade (I/R + CGRP8-37, Group 8) or NADPH oxidase inhibition (I/R + Apocynin, Group 9) on I/R-induced leukocyte rolling (Upper Panel) and adhesion (Lower Panel). Treatment with CGRP8-37 prevented postischemic leukocyte rolling but did not abrogate the development of stationary leukocyte adhesive responses to I/R. Interestingly, we employed the same dose of CGRP8-37 to prevent the anti-adhesive effects of preconditioning with exogenous CGRP or ethanol administered 24 hrs prior to I/R (16), suggesting that the dose of the receptor antagonist was sufficient to effectively block the effects of the neuropeptide. Apocynin treatment also prevented the increase in leukocyte rolling induced by I/R and attenuated the postischemic rise in leukocyte adhesion (Figure 3).

The data depicted in Figure 4 demonstrates that exogenous administration of Ang II by intraperitoneal injection (Ang II, Group 10), in lieu of I/R, induces significant increases in leukocyte rolling (open bars) and adhesion (closed bars). In contrast to the pattern noted after I/R, blockade of CGRP receptors with CGRP8-37 (Ang II + CGRP8-37, Group 11) prevented the increases in both leukocyte rolling and stationary leukocyte adhesion induced by Ang II. NADPH oxidase inhibition attenuated both leukocyte rolling and adhesion induced by Ang II (Ang II + Apocynin, Group 12).

## DISCUSSION

The results of this study provide two lines of evidence implicating the renin-angiotensin system in the proinflammatory response to intestinal I/R. First, the postischemic increases in leukocyte rolling and adhesion were largely abolished by concomitant treatment with $AT_1$ (Valsartan) plus $AT_2$ (PD123,319) receptor antagonists. Second, inhibition of chymase, an important enzymatic source of Ang II especially in injured vasculature, also abrogated I/R-induced leukocyte rolling and adhesion. ACE inhibition or independent blockade of $AT_1$ or $AT_2$ receptors were also effective in preventing postischemic leukocyte rolling, but failed to modify stationary adhesive responses to I/R. Other significant new findings pertain to evidence supporting a role for calcitonin gene-related peptide (CGRP) and NADPH oxidase in I/R-induced, Ang II-mediated leukocyte/endothelial cell adhesive interactions.

Our results contribute to a growing body of evidence indicating that the renin-angiotensin system contributes to I/R-induced inflammatory responses (3, 4, 7, 10, 24, 25, 27). For example, Riaz et al (27) reported that large increases in circulating Ang II concentrations, colonic CXC chemokine expression, and ACE mRNA levels occur after 2 hrs of reperfusion following a 30 min occlusion of the superior mesenteric artery. Petnehazy et al (24) showed that SMA occlusion for 45 min induces $AT_1$ receptor expression, leukocyte rolling, and stationary leukocyte adhesion in small intestinal venules, when assessed 4 hours after reperfusion was initiated. Interestingly, a similar pattern of response was noted in studies conducted in chimeric mice that express $AT_1$ receptors on the vessel wall, but not circulating cells. This latter result suggests that engagement of $AT_1$ receptors on circulating cells vs those expressed in postcapillary

venules plays a more dominant role in eliciting the pro-inflammatory state induced by intestinal I/R. The role of $AT_2$ receptors was not evaluated in either study.

The aforementioned observations support a role for Ang II, acting via $AT_1$ receptors, in I/R-induced stationary leukocyte adhesion but not in the weaker adhesive interactions associated with leukocyte rolling in the small and large intestine. In stark contrast, our work indicates that two mechanistically distinct approaches that interfere with the actions of Ang II (chymase inhibition and combined $AT_1$/$AT_2$ receptor blockade) prevented the increases in both leukocyte rolling and stationary adhesion induced by I/R. On the other hand, we report that ACE inhibition and independent blockade of $AT_1$ or $AT_2$ receptors largely abolished postischemic leukocyte rolling without influencing the increase in stationary leukocyte adhesion induced by I/R. Our findings are consistent with the observations of Piqueras et al (25) who showed that 1 hr exposure of the mesentery to exogenous Ang II at subpressor levels induces leukocyte rolling and adhesion via $AT_1$ and $AT_2$ receptor-dependent expression of P-selectin.

Prior to the present work, the enzymatic source for Ang II in the small intestine and the possible contribution of $AT_2$ receptors in the development of this pro-inflammatory phenotype were largely unknown. While ACE is a major generator of Ang II under normal conditions and in many hypertensive states, a number of recent studies have pointed to the potential involvement of mast cell chymase as another enzymatic source of this peptide in the vessel wall, especially at sites of vascular injury (9, 13). In normal tissues, chymase is stored in mast cells in an inactive form and angiotensin converting enzyme plays a dominant role in Ang II formation. However, chymase acquires the ability to form Ang II following secretion from degranulating mast cells and can become

the predominant source of Ang II in injured tissues. Importantly, this enzyme can account for as much as 80-90% of Ang II formation in the heart and is a significant source of the peptide in damaged arterial vessels (32). Because mast cell density is very high in gastrointestinal tissues and these perivascular cells play a prominent role in the inflammatory response to I/R in the small bowel (17), we sought to determine the relative contribution of chymase vs ACE as potential sources of Ang II in postischemic intestine. Our results indicate that ACE inhibition with Captopril completely prevented the postischemic increase in leukocyte rolling, without influencing stationary adhesive interactions induced by I/R. In contrast, chymase inhibition largely abolished both postischemic leukocyte rolling and adhesion. These results suggest that chymase-generated Ang II may play a more dominant role in inducing the cellular changes that promote stationary leukocyte adhesion, while both ACE- and chymase-dependent generation of Ang II are important for I/R-induced leukocyte rolling. It is possible that ACE may generate Ang II that is more readily accessible to circulating leukocytes while chymase derived Ang II distributes preferentially to the vascular wall. The latter postulate may also explain the apparent discrepancies noted in the studies above (ref. 24,27 vs ref. 25, present study), because ACE was targeted for inhibition and only $AT_1$ receptors were blocked in some studies (24, 27), without examination of the effect of chymase or combined $AT_1/AT_2$ receptor blockade (present study). Differences in time of reperfusion or Ang II exposure (2 and 4 hrs in data from ref. 24, 27 vs 1 hr from ref 25, present study) over which the responses were evaluated may also explain the divergent results.

In addition to catalyzing the formation of Ang II, ACE can also contribute to the formation of bradykinin, a neuropeptide that exhibits both pro- and anti-inflammatory properties, depending on its concentration (29). Since postischemic leukocyte rolling was inhibited by $AT_1$ and/or $AT_2$ receptor blockade, as well as by ACE inhibition, it is most likely that the beneficial actions of ACE inhibition are related to prevention of Ang II, rather than bradykinin formation.

A significant new finding of the present study is that $AT_2$ receptor blockade was as effective as $AT_1$ receptor antagonism in attenuating postischemic leukocyte rolling, while combined blockade of both $AT_1$ plus $AT_2$ receptors was required to prevent I/R-induced leukocyte adhesion. This observation provides the first evidence supporting a role for $AT_2$ receptor engagement in provoking I/R-induced leukocyte/endothelial cell adhesive interactions. Our observations are consistent with the fact that exogenously applied Ang II increases leukocyte rolling and adhesion in the colon and mesentery in the absence of I/R by a mechanism that involves $AT_1$ and $AT_2$ receptor-dependent P-selectin expression (25, 27).

Demonstrating a proadhesive role for $AT_2$ receptor engagement in I/R was somewhat surprising in light of several studies implicating antagonistic effects of $AT_1$ and $AT_2$ in vascular inflammation (4, 5, 14, 15, 18, 22). $AT_2$ receptor activation may induce leukocyte adhesion in intestinal I/R by three possible mechanisms. The first involves the potential generation of proinflammatory reactive nitrogen oxide species formed secondary to NO/superoxide interactions initiated by $AT_2$-dependent eNOS activation coincident with $AT_1$ receptor mediated stimulation of NADPH oxidase (5, 12, 28). Another potential mechanism is suggested by the fact that neutrophils isolated

from hypercholesterolemic patients and exposed to Ang II demonstrate an oxidative response that is more dependent on $AT_2$ receptors, while those isolated from normal patients depend on $AT_1$ receptor activation (23). Thus, I/R may induce a phenotypic change in circulating neutrophils such that $AT_2$ receptor activation contributes to the oxidative burst in these cells. A third, and perhaps more likely, mechanism is suggested by the observations that both oxidants and NO can activate capsaicin-sensitive neurons to release CGRP, a proadhesive peptide well known for its role in neurogenic inflammation (1, 31). In this regard, it is important to note that Ang II increases circulating CGRP levels and CGRP receptor expression (20, 35). Moreover, perivascular nerves, some of which may be capsaicin-sensitive, express $AT_2$ receptors (33). Our results indicating that CGRP receptor blockade attenuates leukocyte rolling and adhesion induced by I/R or exogenously applied Ang II suggest that this inflammatory peptide plays a critical role in producing these adhesive interactions.

The proinflammatory effects of $AT_1$ receptor engagement are well-known and appear to involve activation of NADPH oxidases expressed by endothelial cells, vascular smooth muscle, adventitial fibroblasts and leukocytes during early reperfusion (30). Our work is consistent with this concept in that treatment with a specific NADPH oxidase inhibitor (apocynin) attenuated postischemic leukocyte rolling and adhesion, a finding that corroborates our earlier work demonstrating a similar effect with another NADPH oxidase inhibitor, PR-39 (19). In addition, $AT_1$ receptor activation is associated with a reduction in extracellular superoxide dismutase (5), an effect which would serve to increase the cytotoxicity of oxidants produced at the cell surface. However, $AT_1$

receptor-stimulated leukocyte adhesion occurs by a superoxide-independent mechanism when assessed after 4 hrs of reperfusion (24).

It is of interest to note that several groups have reported that Ang II-induced leukocyte adhesion is not confined to postcapillary venules but also occurs in arterioles when assessed after 4 hrs of exposure to exogenous Ang II, but not when evaluated after 1 hr of exposure (3, 21). Our results are consistent with the latter observations and suggest that arteriolar adhesion requires a more prolonged exposure to Ang II than the 1 hr exposure protocol employed in the present study.

In summary, the results of this study demonstrate that Ang II derived from the enzymatic activity of chymase, as well as ACE, contributes to the proadhesive phenotype demonstrated by postcapillary venules after intestinal I/R. The increased numbers of rolling and adherent leukocytes that occur secondary to I/R-induced Ang II formation appears to be mediated by an $AT_1$ and $AT_2$ receptor-dependent mechanism that involves release of CGRP and generation of oxidants derived from NADPH oxidase.

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## FIGURE LEGENDS

**Figure 1.** Effects of ischemia/reperfusion alone (I/R) or I/R plus treatment with an angiotensin $AT_1$ receptor antagonist (I/R + Valsartan), an $AT_2$ receptor antagonist (I/R + PD123,319), or combined $AT_1$ plus $AT_2$ receptor blockade (I/R + Valsartan + PD123,319) on postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion. * indicates mean values that were statistically different from the sham control group at $p<0.05$.

**Figure 2.** Effects of ischemia/reperfusion alone (I/R) or I/R plus treatment with inhibitors of angiotensin converting enzyme (I/R + Captopril) or chymase (I/R + Y-40079) on postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion. * indicates mean values that were statistically different from the sham control group at $p<0.05$.

**Figure 3.** Effects of ischemia/reperfusion alone (I/R) or I/R plus treatment with an calcitonin gene-related peptide receptor antagonist (I/R + CGRP8-37) or an NADPH oxidase inhibitor (I/R + Apocynin) on postischemic leukocyte rolling (Upper Panel) or stationary leukocyte adhesion (Lower Panel) determined after 30 and 60 min reperfusion. * indicates mean values that were statistically different from the sham control group at $p<0.05$.

**Figure 4.** Effects of exogenous administration of angiotensin II (Ang II) on leukocyte rolling (open bars) and stationary leukocyte adhesion (closed bars) in the absence and presence of CGRP receptor blockade (Ang II + CGRP8-37) or NADPH oxidase inhibition (Ang II + Apocynin). * indicates mean values that were statistically different from the sham control group at $p<0.05$.

Figure 1

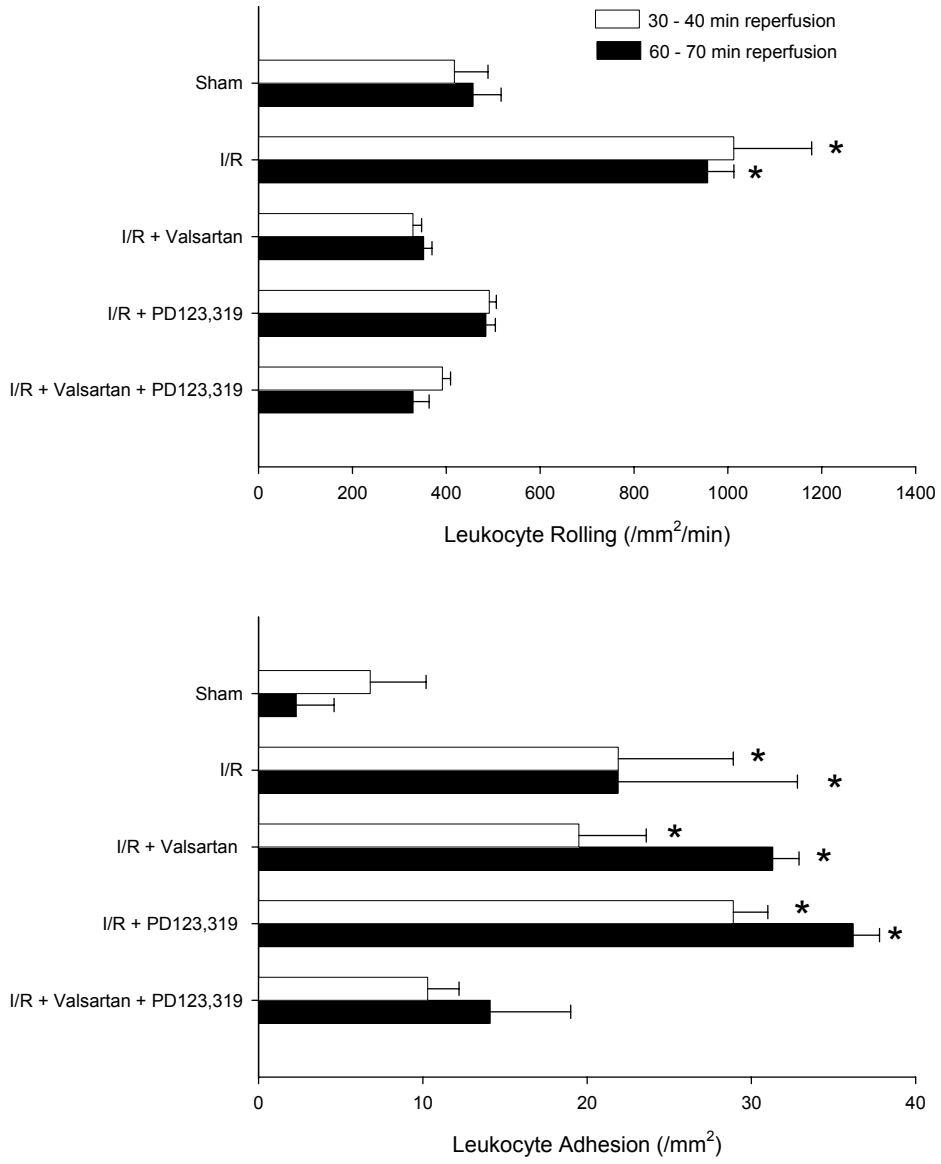

30 - 40 min reperfusion
60 - 70 min reperfusion
Sham
I/R
I/R + Valsartan
I/R + PD123,319
I/R + Valsartan + PD123,319
0
200
400
600
800
1000
1200
1400
Leukocyte Rolling (/mm2/min)
Sham
I/R
I/R + Valsartan
I/R + PD123,319
I/R + Valsartan + PD123,319
0
10
20
30
40
Leukocyte Adhesion (/mm2)

Figure 2

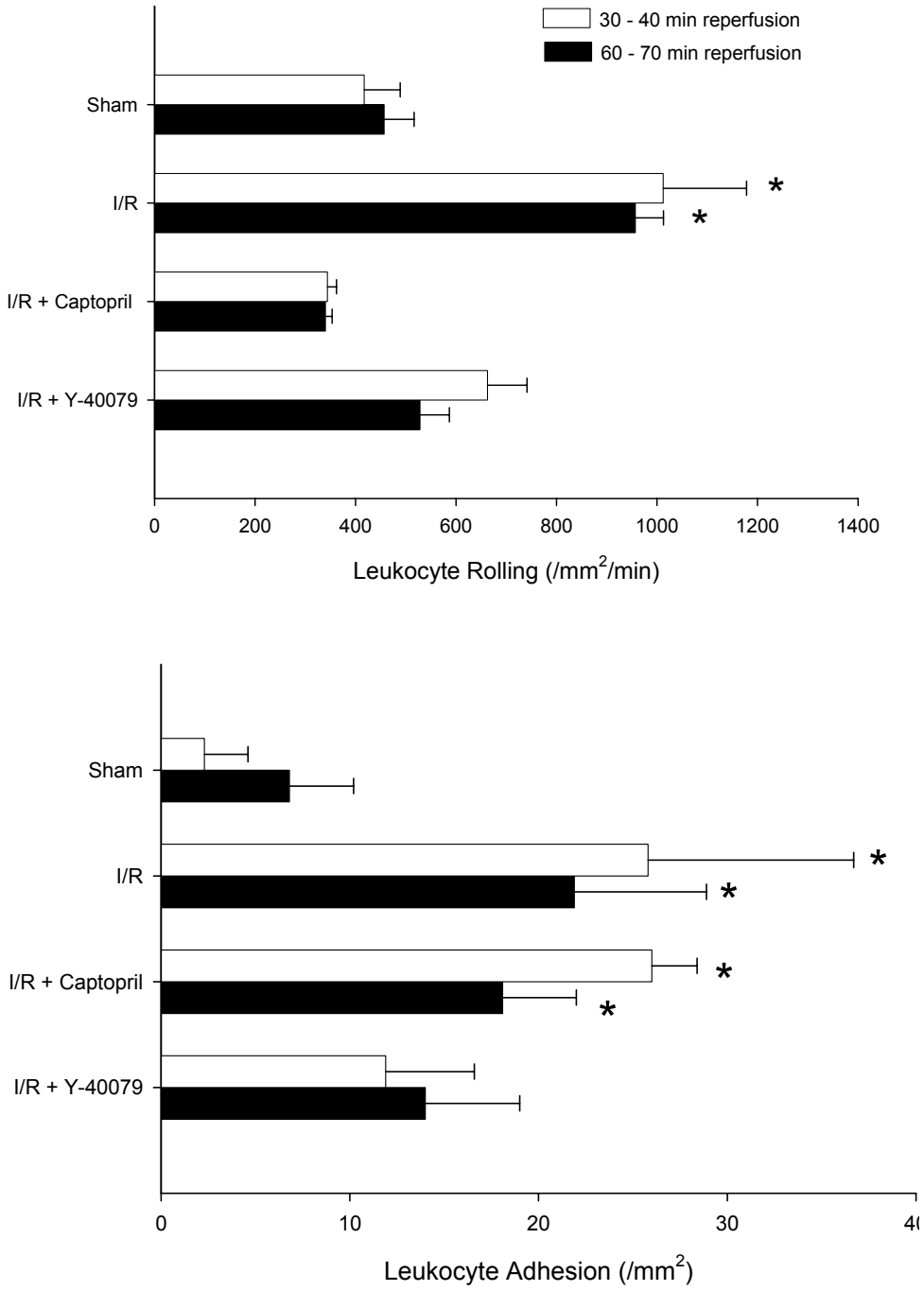
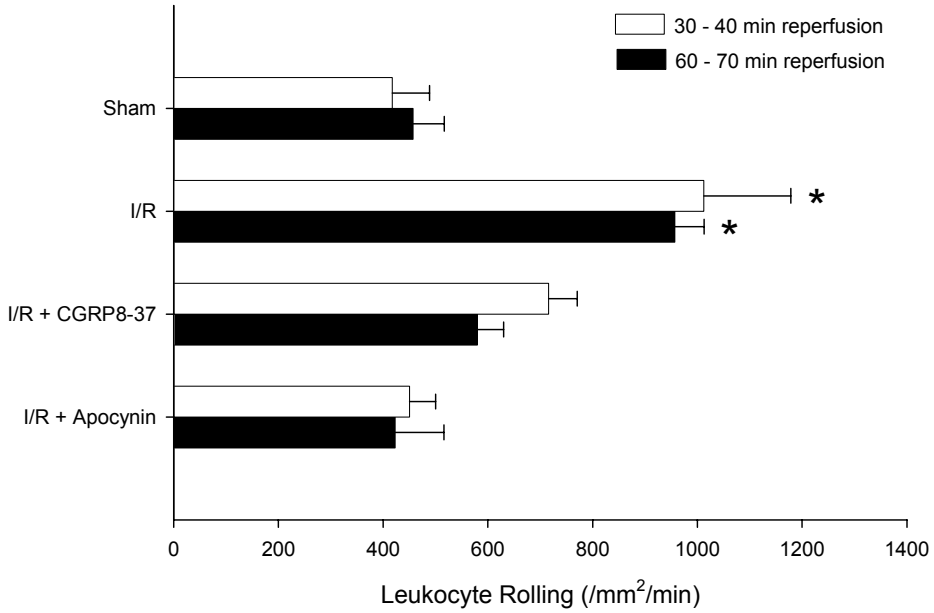

30 - 40 min reperfusion
60 - 70 min reperfusion
Sham
I/R
I/R + Captopril
I/R + Y-40079
*
*
0
200
400
600
800
1000
1200
1400
Leukocyte Rolling (/mm2/min)
Sham
I/R
I/R + Captopril
I/R + Y-40079
*
*
*
*
0
10
20
30
Leukocyte Adhesion (/mm2)

Figure 3


30 - 40 min reperfusion
60 - 70 min reperfusion
Sham
I/R
I/R + CGRP8-37
I/R + Apocynin
*
*
0
200
400
600
800
1000
1200
1400
Leukocyte Rolling (/mm2/min)

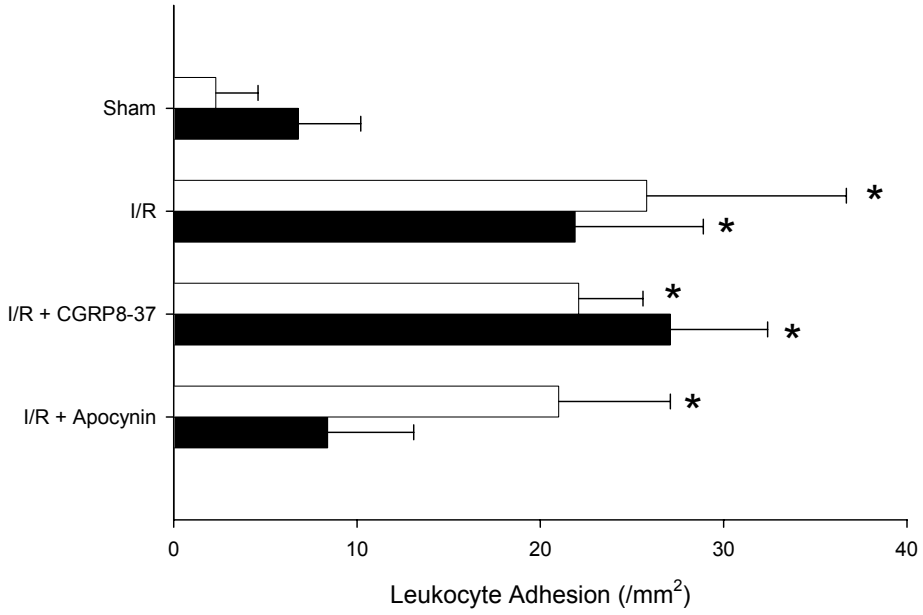
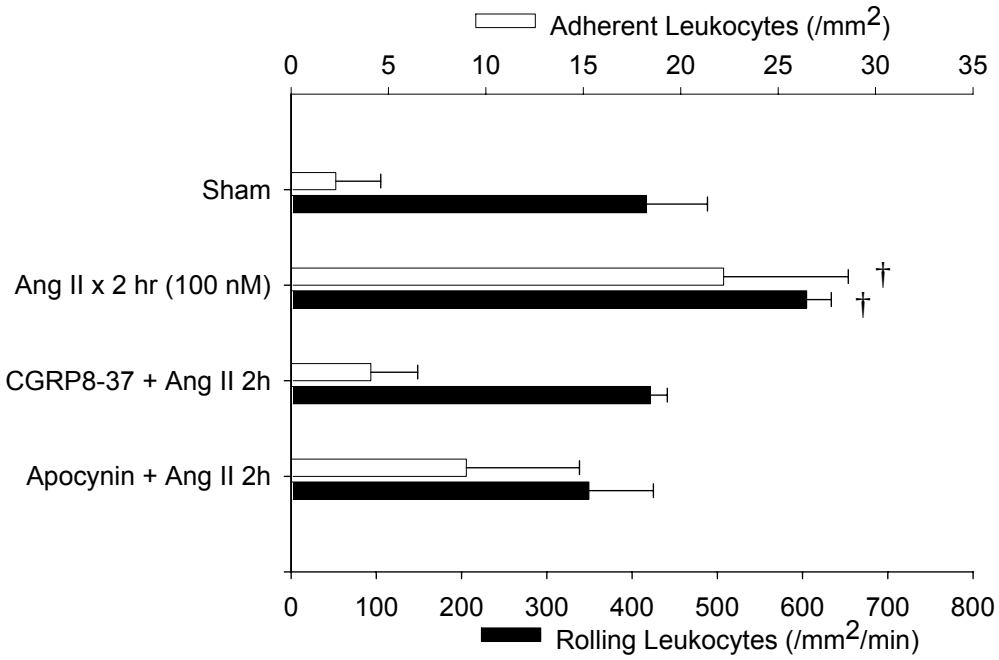

Sham
I/R
I/R + CGRP8-37
I/R + Apocynin
*
*
*
*
*
0
10
20
30
40
Leukocyte Adhesion (/mm2)

Figure 4


Adherent Leukocytes (/mm2)
0
5
10
15
20
25
30
35
Sham
Ang II x 2 hr (100 nM)
†
†
CGRP8-37 + Ang II 2h
Apocynin + Ang II 2h
0
100
200
300
400
500
600
700
800
Rolling Leukocytes (/mm2/min)

## SUMMARY

Although ischemic preconditioning is the strongest form of in vivo protection against ischemia and reperfusion injury (I/R), it is not as feasible in clinical practice as pharmacologic preconditioning. Discovering the molecular mechanisms that mediate ischemic preconditioning can provide us with the ability to modulate and protect against I/R. The in vivo and in vitro data presented in this dissertation were designed to determine the adaptations that occur in postischemic murine small intestine preconditioned with antecedent hydrogen sulfide ($H_2S$).

The rationale and original design for these experiments stems from a close relationship between $H_2S$ and two endogenous gaseous signaling molecules known to act as preconditioning stimuli, nitric oxide (NO) and carbon monoxide (CO) releasing molecules. Moreover, many other preconditioning stimuli also appear to promote the production of NO, but as an initial triggering event in the acquisition of tolerance to I/R , while formation of CO appears to serve as an important effector of protection. $H_2S$ is present in the blood at concentrations in the range of 10-100 $\mu$M, levels that far exceed those reported for NO and CO. Like NO and CO, $H_2S$ produces vasorelaxation, but does so by a mechanism distinct from these gaseous monoxides, activating $K_{ATP}$ channels rather than guanylyl cyclase. However, $H_2S$ can enhance the vasorelaxant effects of NO and, conversely, the production of $H_2S$ can be up-regulated by NO. In addition, preconditioning with NO donors or CO releasing molecules prevents postischemic leukocyte infiltration and exerts infarct-sparing effects in postischemic tissues.

It is well known that leukocytes play a key role in I/R injury. Recognition of the importance of the inflammatory process to the pathogenesis of I/R injury has led to an intensive research effort directed at identifying strategies to prevent leukocyte infiltration into post-ischemic tissues. Indeed, work conducted over the past 20 years has led to the development of the concept that oxidant-induced leukocyte/endothelial cell interactions are largely responsible for the microvascular dysfunction induced by reperfusion. Like NO, $H_2S$ and its major dissociation product $HS^-$, are extremely reactive, most likely exerting biological effects through direct covalent modification of target molecules. On the one hand, $HS^-$ is a free radical species while on the other $H_2S$ is a thiol and can interact with and "scavenge" free radicals. The biochemical potential of $H_2S$ made it an attractive molecule to study in the setting of I/R.

The results of the initial studies provided the first evidence that exposing the small bowel to the $H_2S$ donor, NaHS, induces the development of an anti-inflammatory phenotype in murine small intestine such that postcapillary venules fail to support leukocyte rolling and adhesion when subjected to (I/R) 24 hrs later. Interestingly, treatment with NaHS 1 hr prior to I/R or by continuous superfusion with this donor during reperfusion failed to elicit these anti-adhesive responses. These observations suggest that $H_2S$ is effective at inducing delayed acquisition of tolerance to I/R (late phase preconditioning) but does not instigate early phase preconditioning nor does it exert a direct effect to limit postischemic leukocyte rolling and adhesion when the bowel is exposed to this donor during reperfusion.

Preconditioning with $H_2S$ donors may represent a promising new avenue for prevention of the microvascular complications of I/R.

In view of the powerful anti-adhesive effects of NaHS treatment 24 hrs prior to I/R, but not when administered 1 hr prior to induction of ischemia or during reperfusion, we focused our attention on the mechanisms that may be involved in eliciting late phase preconditioning by this $H_2S$ donor. Because it has been demonstrated that exposing the mesentery to exogenous $H_2S$ attenuates leukocyte rolling and adhesion induced by proinflammatory stimuli such as TNFα, it is tempting to speculate that the protective effects noted were due to the continued presence of sulfides 24 hrs after administration. However, this explanation is highly unlikely because of the inherent instability of $H_2S$ in our experimental conditions owing to oxidation in mitochondria, methylation in the cytosol, and scavenging by metalloproteins, disulfide-containing proteins, and heme compounds. In addition, $H_2S$ is subject to rapid catabolism via thiol S-methyltransferase and rhodenese, both of which are expressed in the small intestine. The aforementioned observations suggest that the protective actions of $H_2S$ preconditioning are initiated by activation of downstream signaling mechanisms, in a manner analogous to other short-lived preconditioning stimuli, such as NO.

Several mechanisms of this protective effect were implicated by the studies outlined in this dissertation. Initially, in vitro studies were employed to determine if $H_2S$ upregulates the expression of various proteins implicated in the molecular mechanisms of ischemic preconditioning. Additionally, the time course of this effect was studied in vitro to characterize the effects of these triggers and/or mediators.

The cell culture model chosen was as closely relevant to the in vivo studies as is possible with any cell culture model. Human microvascular endothelial cells (HMEC-1) were used to most closely represent the protein expression that might regulate leukocyte-endothelial interactions measured in our in vivo studies.

A number of recent studies implicate the activation of a number of kinases, such as p38 MAPK, in the development of preconditioned states. At least 6 different isoforms of p38 MAPK have been described. This MAPK is activated by dual phosphorylation on a Thr-Gly-Tyr motif in response to a number of stimuli implicated as triggers for preconditioning, including antecedent exposure to short bouts of ischemia or NO donors. Additionally, since $H_2S$ has been shown to increase p38 MAPK phosphorylation, we postulated that the anti-inflammatory effects induced by exogenous $H_2S$ might be elicited through activation of this kinase. The time course study used to evaluate the expression of phosphorylated p38 MAPK and endothelial nitric oxide synthase (eNOS) in western blots from human microvascular endothelial cells exposed to NaHS showed upregulated expression within 1 and 10 min, peaking at 1-6 hrs and still greater than control values at 24 hrs. These observations led us to postulate that NaHS exposure may trigger the acquisition of delayed or late phase tolerance to I/R by activating p38 MAPK and eNOS.

Our results show that pharmacologic inhibition of p38 MAPK by either of SB203580 or SK80062 abolished the protective effects of late phase NaHS-PC to limit leukocyte rolling and leukocyte adhesion induced by I/R 24 hrs after preconditioning. Although $H_2S$ does not appear to produce vasodilation by a NOS-dependent mechanism, $H_2S$ can enhance the vasorelaxant effects of NO and,

conversely, the production of $H_2S$ can be up-regulated by NO. Thus, we evaluated the role of eNOS in the vasculoprotection afforded by late phase $H_2S$-PC by employing a pharmacologic inhibitor approach in WT animals and by use of an eNOS knockout model. The ability of $H_2S$-PC to prevent postischemic leukocyte rolling was completely absent in eNOS-deficient mice and was markedly attenuated by NOS inhibition with L-NIO in wild-type animals. It is unclear whether $H_2S$ directly activates eNOS and/or p38 MAPK or targets other molecular elements upstream from these signaling enzymes. $H_2S$ can induce dithiol reduction and ligand displacement from heme iron, posttranslational protein modifications that may subserve this function. In addition, $H_2S$ can interact with the oxygen binding site of cytochrome c oxidase in the respiratory chain and leads to enhanced mitochondrial production of reactive oxygen species (ROS).

Since the vasodilator actions of $H_2S$ are mediated by activation of $K_{ATP}$ channels and antecedent treatment with $K_{ATP}$ channel openers exerts infarct-sparing effects, prevents capillary no-reflow, and attenuates neutrophil infiltration in tissues subsequently exposed to I/R, we hypothesized that entrance into the anti-inflammatory phenotype induced by $H_2S$ preconditioning might be initiated by activation of potassium channels. The results presented are the first evidence that NaHS induces the development of an anti-inflammatory phenotype in postcapillary venules through both mitochondrial and plasmalemmal-specific channel activation. Additionally, we show that the plasmalemmal-specific $K_{ATP}$ channel activator preconditions the mesenteric postcapillary venules in lieu of $H_2S$-PC.

Potassium channels are ubiquitously expressed cell membrane proteins that participate in a wide variety of physiological processes, including regulation of vasomotor tone, heart rate, neurotransmitter release and muscle contraction. Given the broad range of functional activities, it is not surprising that a diverse spectrum of functionally distinct potassium channels have evolved. With regard to preconditioning, ATP-sensitive potassium channels have received the most attention. However, the results of several recent studies point to the importance of another set of potassium channels, the large conductance, calcium-activated potassium ($BK_{Ca}$) channels, as critical triggers for the development of the protected phenotype in response to preconditioning stimuli. These channels are expressed by parenchymal cells in a variety of organs, but also in vascular tissues, including endothelial and vascular smooth muscle cells. The present study provides strong support for the notion that NaHS-PC activates $BK_{Ca}$ channels in endothelial cells, which play a critical role in the development of an anti-inflammatory phenotype in the murine small intestine, such that postcapillary venules fail to support increased leukocyte rolling and adhesion induced by exposing the bowel to I/R 24 hrs after application of this preconditioning stimulus. Three lines of evidence support this conclusion. First, coincident administration of the $BK_{Ca}$ channel inhibitor paxilline with NaHS 24 hrs prior to I/R effectively abolished the effects of NaHS-PC to prevent postischemic leukocyte rolling and adhesion. Second, the anti-inflammatory effects of NaHS-PC were mimicked by preconditioning with the $BK_{Ca}$ channels activator NS-1619. Third, we provided evidence that exposing cultured endothelial cells to NaHS

at the same concentration achieved in our in vivo studies, activates an iberiotoxin-sensitive current.

Our work demonstrated roles for NaHS-dependent eNOS, p38-MAPK, $K_{ATP}$, and $BK_{Ca}$ channel activation as critical triggering elements that inaugurate or trigger entrance into a preconditioned anti-inflammatory state. However, the identity of the effector(s) or mediator(s) of the anti-adhesive effects of NaHS-PC were yet largely unexplored. $H_2S$ upregulates the expression of HO-1 and was of particular interest to us in the development of the experiments because the catalytic products of HO-1-mediated heme metabolism exhibit powerful anti-oxidant and anti-adhesive properties. Since I/R induces oxidant-dependent leukocyte rolling and adhesion, we hypothesized that NaHS-PC may be mediated by upregulated expression of this heat shock protein.

To begin to address this postulate, we evaluated the effect of $H_2S$ donor treatment on HO-1 in the small intestine and vascular tissue. We then turned our attention to the possibility that treatment with a non-isoform selective pharmacologic inhibitor of heme oxygenase would prevent the anti-inflammatory effects of NaHS-PC when administered during I/R 24 hrs later. Pharmacologic inhibition of heme oxygenase with tin protoporphyrin, SnPPIX, but not the inactive protoporphyrin, copper protoporphyrin, CuPPIX, completely prevented the anti-adhesive actions of antecedent NaHS. We also demonstrated that preconditioning with an agent known to upregulate the expression and activity of HO-1 (hemin), 24 hrs prior to I/R was as effective as antecedent NaHS in preventing postischemic leukocyte rolling and adhesion.

While the aforementioned studies provide strong support for the notion that heme oxygenase serves as an effector of NaHS-PC, the pharmacologic inhibitor studies fail to identify the heme oxygenase isoform responsible for these anti-inflammtory actions. In addition, the studies rely on the specificity of SnPPIX as a heme oxygenase inhibitor. Indeed, there is evidence suggesting that this agent also inhibits iNOS, an enzymatic source of NO, a gaseous monoxide that not only exerts anti-adhesive effects but has also been implicated in other forms of preconditioning. In light of these issues, we evaluated the effectiveness of NaHS as a preconditioning stimulus in HO-1 knockout mice and demonstrated that the anti-adhesive effects antecedent treatment with this $H_2S$ were completely absent. Taken together, our studies provide compelling support for the notion that HO-1 is a critically important molecular end-effector of the anti-adhesive phenotype elicited by NaHS-PC.

Previous studies confirm that $H_2S$ also exerts antioxidant effects and limits oxidative stress by virtue of its actions to raise intracellular glutathione levels by enhancing the activity of γ-glutamylcysteine synthetase and upregulating cysteine transport. Additionally, $H_2S$ enhances the ability of superoxide dismutase to scavenge superoxide. As preliminary work to determine whether these antioxidant actions of hydrogen sulfide are relevant to current work in the field of cardiovascular disease and angiotensin-derived reactive oxygen species, we studied the role of angiotensin II in our model of murine I/R. Two lines of evidence implicated the renin-angiotensin system in the proinflammatory response to intestinal I/R. First, the postischemic increases in leukocyte rolling and adhesion were largely abolished by concomitant treatment with $AT_1$ (Valsartan) plus $AT_2$ (PD123,319) receptor

antagonists. Second, inhibition of chymase, an important enzymatic source of Ang II especially in injured vasculature, also abrogated I/R-induced leukocyte rolling and adhesion. ACE inhibition or independent blockade of $AT_1$ or $AT_2$ receptors were also effective in preventing postischemic leukocyte rolling, but failed to modify stationary adhesive responses to I/R. Other significant new findings pertain to evidence supporting a role for calcitonin gene-related peptide (CGRP) and NADPH oxidase in I/R-induced, angiotensin II-mediated leukocyte/endothelial cell adhesive interactions. Our results contribute to a growing body of evidence indicating that the renin-angiotensin system (RAS) contributes to I/R-induced inflammatory responses. Future studies will involve the investigation of whether $H_2S$-PC mediates the effects of the RAS and thereby could contribute to protection in hypertensive patients, a class of patients with cardiovascular disease and pathology specifically relevant to dysregulation of RAS and susceptible to $H_2S$-PC. Specific emphasis would include additional analysis of the systemic pressure effects of $H_2S$-PC.

In summary, we have elucidated important mechanistic distinctions that underlie the development of a preconditioned phenotype in response to antecedent $H_2S$. Acute exposure to physiologically relevant doses of $H_2S$, through $H_2S$ donor (NaHS) treatment, prevents postischemic leukocyte rolling and adhesion by a mechanism that is triggered by eNOS, p38 MAPK, mitochondrial and plasmalemmal $K_{ATP}$ channels, and $BK_{Ca}$ channels. These initiating events culminate in the expression of HO-1 as an effector mechanism during reperfusion secondary to $H_2S$-PC. Future studies will be directed at determining the temporal relationship of these triggers, mediators and end effector molecules. With these future studies, a schema of the

molecular events following $H_2S$-PC would emerge, detailing the effects of $H_2S$ from the moment the small intestine is exposed to the preconditioning stimulus through the machinery that is activated to protect against I/R, the endogenous mediators of the preconditioning stimulus and finally ending with identification of effector molecules.

Altogether, the relevance of $H_2S$-PC can be applied to the effects of this preconditioning stimulus on endogenous modulators of I/R, as well as those that are already targeted in patients with cardiovascular disease, for example, angiotensin II-mediated cardiovascular risk that is treated with ACE inhibitors. The evidence presented in this dissertation provides the first indication that $H_2S$ can modify reversible I/R injury and extends our knowledge of this gaseous mediator's spectrum of biological activity. This work provides an impetus for further exploration of the $H_2S$ pathway in cardiovascular regulation and its specific roles in ischemia reperfusion syndromes.

## “FROM BENCH TO BEDSIDE”

An interesting question was asked during the oral defense of this dissertation, and deserves recapitulation in this written document: “Can you design an experiment where you could integrate the information you have learned with these in vivo and in vitro studies into a separate and second clinically-relevant study?” – Kevin Dellsperger, MD, PhD, Professor and Chairman of the Department of Internal Medicine, University of Missouri – Columbia.

The protective effects of hydrogen sulfide ($H_2S$) are already proven in clinical practice, to a degree. One agent used as a donor of $H_2S$ is N-acetyl-cysteine (NAC). This agent is used with a wide margin of safety in the treatment of acetaminophen toxicity and before radiologic procedures requiring intravenous contrast in patients with known or suspected kidney damage. NAC is a free radical “sponge” and directly interacts with reactive oxygen species, including superoxide anion, hydrogen peroxide, and hydroxyl radicals. In order to integrate the knowledge obtained from in vitro and in vivo studies showing that hydrogen sulfide could potentially precondition endothelium from ischemia/reperfusion injury, we could employ patients with known coronary artery disease into a clinical trial using NAC, as an $H_2S$-donor, with the end-point measurement of incidence of coronary events as the measured outcome marker. Additional outcome markers could include the evaluation of coronary or non-coronary vascular samples obtained at the time of percutaneous interventions to determine expression of the implicated initiators, mediators, and effector in these studies (i.e. endothelial nitric oxide synthase, p38 MAPK, $K_{ATP}$

channels, $BK_{Ca}$ channels, heme oxygenase, and angiotensin II). Eventually, other patient populations could be studied and compared to control and coronary artery disease populations. The patient populations that seem particularly interesting in regards to the mechanism of development of ischemia and reperfusion injury, include those disease states that are associated with increase oxidative stress and a predisposed risk for myocardial infarction: patients with diabetes and hyperlipidemia. The outcome markers could be compared with plasma $H_2S$, nitric oxide, and carbon monoxide levels yielding a clearer relevance of how these three gaseous signaling molecules interact with each other in clinically relevant syndromes of ischemia/reperfusion. Altogether, a clinical trial designed with the use of NAC as a preconditioning stimulus is justified in terms of its safety index, as well as its pathophysiological significance in relation to $H_2S$ as an endogenous gaseous signaling molecule.

UNPUBLISHED DATA

Figure A. Neither betacyanoalanine (BCA) nor propargylglycine (PPG) (both are CSE inhibitors) were effective at attenuating EPC against LR or LA after 60 minutes of reperfusion.

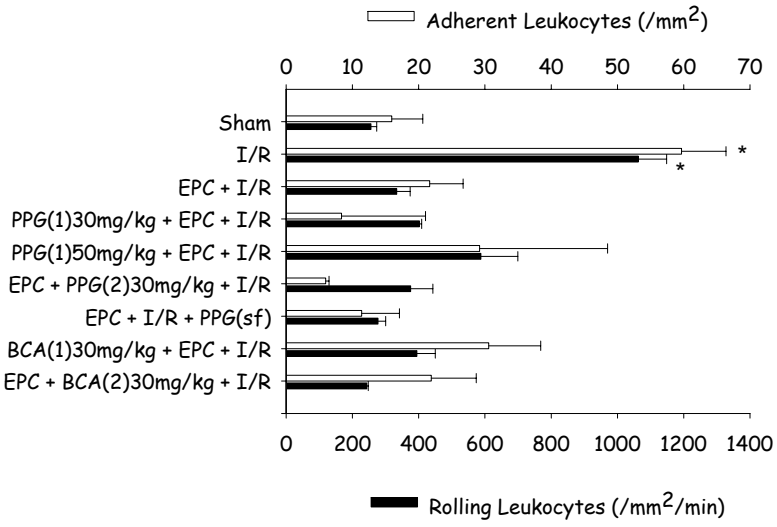
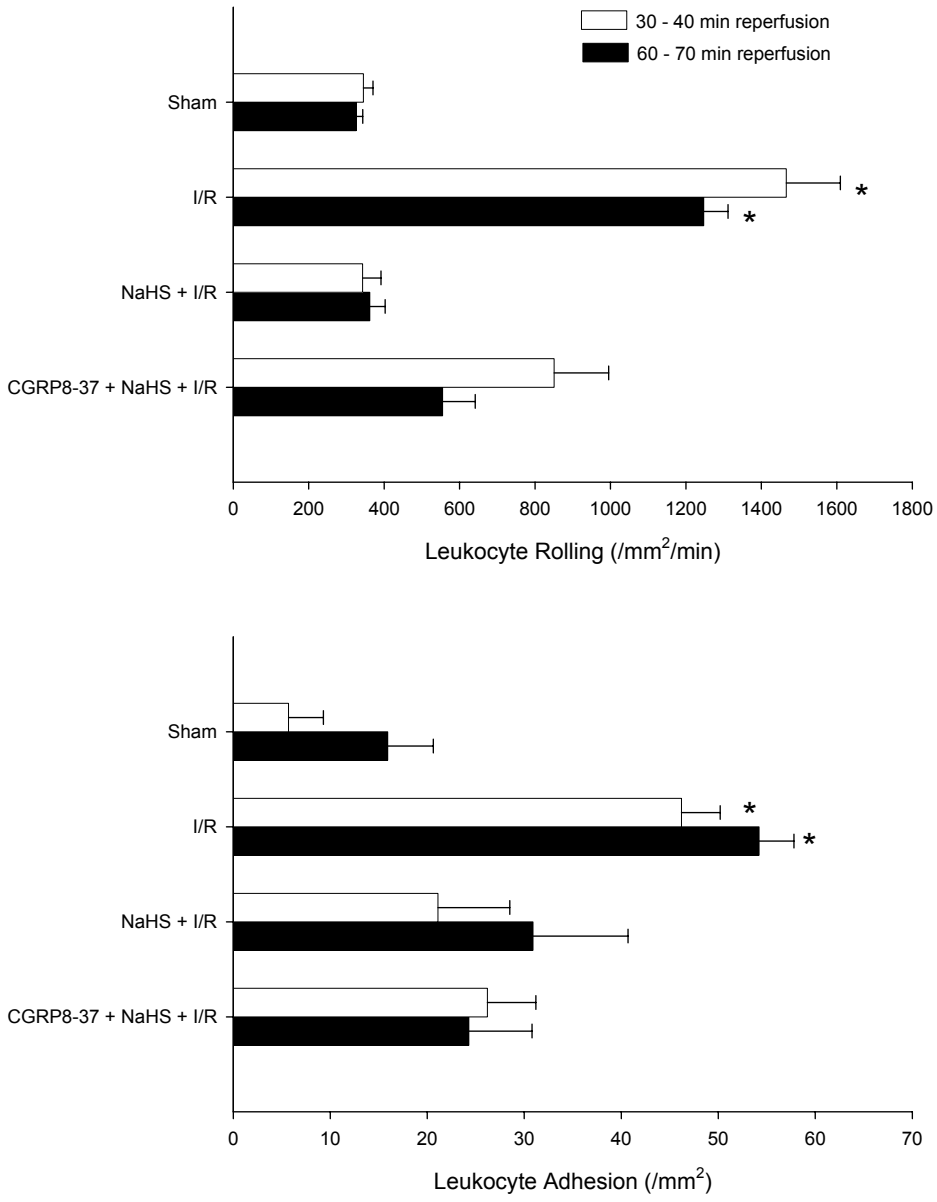


Figure B. CGRP inhibition did not attenuate the effects of NaHS-PC



# VITA

Mozow Yusof was born on June 8, 1976 in Kabul, Afghanistan. She obtained her primary education in Kansas City, Missouri, and secondary education in Lenexa, Kansas, where she graduated from Shawnee Mission Northwest High School in May 1994. She continued her education, studying Life Sciences at the University of Missouri – Rolla. After obtaining her Bachelor of Science degree in May 1997, she began graduate studies at the University of Missouri – Rolla as a Master of Science candidate in the Chemistry Department. She studied under the only female faculty member of the department, and only biochemist, Dr. Nuran Ercal, MD, PhD. Her thesis work was titled, "Plausible antioxidant functions of N-acetylcysteine and D-penicillamine in the treatment of copper toxicity." After completing her Masters degree, Mozow returned to Kansas City to work as an analytical chemist at Quintiles, Inc., a pharmaceutical contract company. After one year as a chemist analyzing drug samples for clinical use, Mozow returned to academia and the study of clinical medicine, and began studying at the University of Missouri – Columbia School of Medicine as a dual-degree candidate for her MD and PhD degrees. Mozow completed the MD degree in 2007, just before defense of the PhD degree. Mozow stayed at the University of Missouri – Columbia for clinical training in Internal Medicine, with her future goals directed at a career in academic medicine and the study of cardiovascular disease.