Thiol Oxidation Activates a Novel Redox-Regulated Coronary Vasodilator Mechanism Involving Inhibition of Ca^{2+} Influx

Takafumi Iesaki, Michael S. Wolin

Abstract—This study examines the mechanism of relaxation of isolated endothelium-removed bovine coronary arteries (BCAs) to the thiol oxidant diamide. BCAs precontracted with KCl or the thromboxane A_2 receptor agonist U46619 showed a concentration-dependent reversible relaxation on exposure to 10 μmol/L to 1 mmol/L diamide. This relaxation was enhanced by an inhibitor of glutathione reductase, and it was not altered by severe hypoxia, the presence of inhibitors of soluble guanylate cyclase, K^+ channels, tyrosine kinases, or probes that modulate levels of superoxide. The relaxation was almost eliminated when BCAs were precontracted with a phorbol ester that causes a contraction that is largely independent of extracellular Ca^{2+}. The initial transient contraction elicited by 5-hydroxytryptamine in Ca^{2+}-free solution was not altered by the presence of 1 mmol/L diamide; however, a subsequent tonic contraction on addition of $CaCl_2$ was inhibited by diamide. Diamide also inhibited contractions caused by the addition of $CaCl_2$ to Ca^{2+}-free Krebs' buffer containing Bay K8644 (an L-type Ca^{2+} channel opener) or KCl. Relaxation to diamide was attenuated by L-type Ca^{2+} channel blockers (nifedipine and diltiazem). Thus, thiol oxidation elicited by diamide appears to activate a novel redox-regulated vasodilator mechanism that seems to inhibit extracellular Ca^{2+} influx. **(*Arterioscler Thromb Vasc Biol.* 2000;20:2359-2365.)**

Key Words: calcium ■ diamide ■ redox signaling ■ thiol redox ■ vasodilation

Intracellular thiols are known to be a major cellular defense mechanism for oxidant stress, and changes in cellular redox status have been proposed to play a role in vascular tone responses. There is evidence that alterations in the thiol redox of key sites on regulatory proteins is a potential mechanism of controlling the function of these systems in a signaling-like manner.[1] The thiol oxidant diamide is known to promote the reversible oxidation of glutathione (GSH) and adjacent protein thiols (RSH) to their disulfide forms [GSSG and R(S-S)] and to promote the S-thiolation (eg, RSSG) of reactive protein thiols.[2] Diamide was initially observed by Weir et al[3] to cause vasodilation in the precontracted pulmonary circulation. This observation evolved into studies showing that oxidants opened plasma membrane K^+ channels in pulmonary arterial smooth muscle through a mechanism potentially mediated through thiol oxidation.[4] These studies also contributed to the development of a hypothesis for the involvement of thiol redox regulated K^+ channels in the process in pulmonary hypoxic vasoconstriction.[4,5] There is also substantial evidence that soluble guanylate cyclase (sGC) is an additional vasodilator mechanism that could be controlled by interactions between oxidants and thiol redox.[6–8] The oxidation of GSH is also likely to increase the levels of intracellular peroxides and reactive hydroxyl radical–like species derived from peroxides in a manner that could enhance the magnitude of the signaling actions of these species. Multiple additional cellular control systems, such as tyrosine phosphorylation–initiated activation of the G protein $p21^{ras}$, phospholipases A_2, C, and D, protein kinase C, and mitogen-activated protein kinases,[9–11] key enzymes in energy metabolism,[12] and systems that control the storage and release of Ca^{2+}[13,14] could be regulated by processes linked to oxidants and thiol redox. Thus, thiol redox could control multiple systems that are involved in the regulation of vascular force.

In the present study, we examined the mechanism through which diamide causes relaxation of isolated endothelium-removed bovine coronary arteries (BCAs). Endothelium was removed to avoid the complicating effects of redox changes on endothelial mediator release. The present study began with investigating the role of stimulation of increases in sGC activity, K^+ channel opening, and reactive O_2 species in the mechanism of relaxation of BCAs elicited by diamide because of the existing evidence[1] that these processes could potentially control vascular contractile function through a regulatory mechanism normally modulated by changes in thiol redox. However, additional regulatory mechanisms po-

Received June 29, 2000; revision accepted July 21, 2000.

From the Department of Physiology, New York Medical College, Valhalla, NY.

Presented in part at the Experimental Biology '99 Meeting, Washington, DC, April 17–21, 1999, and published in abstract form (*FASEB J.* 1999;13:A198) and at the 72nd Scientific Sessions of the American Heart Association, Atlanta, Ga, November 7–10, 1999, and published in abstract form (*Circulation.* 1999;100[suppl I]:I-418).

Correspondence to Michael S. Wolin, PhD, Department of Physiology, New York Medical College, Valhalla, NY 10595. E-mail mike__wolin@nymc.edu

***Arterioscler Thromb Vasc Biol.* is available at http://www.atvbaha.org**

tentially influenced by thiol redox had to be explored when the initial studies did not detect a role for these mechanisms in the relaxant actions of diamide. The subsequent detection of evidence of a role of inhibiting Ca^{2+} influx in the relaxation to diamide resulted in a characterization of the properties of the processes that were involved.

Methods

Materials

Many of the reagents used in the present study were obtained from sources previously described.[15–19] Diamide, dithiothreitol (DTT), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), tetraethylammonium chloride (TEA), charybdotoxin (CTX), glibenclamide, 4-aminopyridine, genistein, phorbol 12,13-dibutyrate (PDBu), Bay K8644, EGTA, nifedipine, and diltiazem were from Sigma Chemical Co. LY83583 was from Cayman Chemicals.

Measurement of Changes in Force in BCAs

Isolated endothelium-removed left circumflex coronary arterial rings were prepared from slaughterhouse-derived bovine hearts and studied for changes in isometric force, as previously described.[16–18] The rings were incubated in individually thermostated (37°C) 10-mL baths (Metro Scientific) for 2 hours at an optimal passive tension of 5 g in Krebs' bicarbonate buffer (pH 7.4) containing the following (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 1.5, $NaHCO_3$ 25, $MgSO_4$ 1.1, KH_2PO_4 1.2, and glucose 5.6, gassed with 21% O_2/5% CO_2 (balance N_2). After a 2-hour equilibration and a brief depolarization with 123 mmol/L KCl, the BCAs were then allowed to reequilibrate for 30 minutes before the experiments were conducted. After a 30-minute equilibration period, the experiments described in Results were conducted. The doses of contractile agents were selected to generate ≈70% of maximal force (typically ≈14 g of force). In studies examining the actions of mechanistic inhibitors on the response to diamide, probes were usually added together with contractile agents, and they were incubated with BCAs for at least 15 minutes before exposure to increasing cumulative concentrations of diamide or other relaxing agents. BCNU, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), LY83583, glibenclamide, genistein, and nifedipine were dissolved in dimethyl sulfoxide, and the 0.05% to 0.1% concentrations used did not alter the vascular responses. None of the probes or treatments, except those mentioned in Results, had a statistically significant effect on force generation.

Statistical Analysis

Results are expressed as mean±SE, with n equal to the number of animals used. Comparisons between groups were made by ANOVA and Student *t* test with a Bonferroni correction for multiple comparisons. A value of $P<0.05$ was used to determine statistical significance.

Results

Diamide Causes a Reversible Relaxation of BCAs

Figure 1A contains a typical experiment using BCAs initially precontracted with the thromboxane A_2 receptor agonist U46619 (0.1 μmol/L) before exposure to the thiol oxidant diamide. As shown in Figure 1A (top), a dose of diamide (0.3 mmol/L) that oxidizes thiols in cardiovascular preparations[20–22] caused BCAs to relax (74±8% relaxation, n=4) in a manner that was reversed (106±3% recovery) by the thiol reductant DTT (1 mmol/L). DTT did not reverse the relaxation (90±4%, n=6) elicited by stimulation of adenylate cyclase by 1 μmol/L forskolin (−2±4% recovery, Figure 1A, middle). Washing out of the diamide with 37°C Krebs' buffer containing U46619 also caused a rapid reversal (107±2% recovery, n=8) of the relaxation (64±6% relaxation) to diamide (See Figure 1A, bottom).

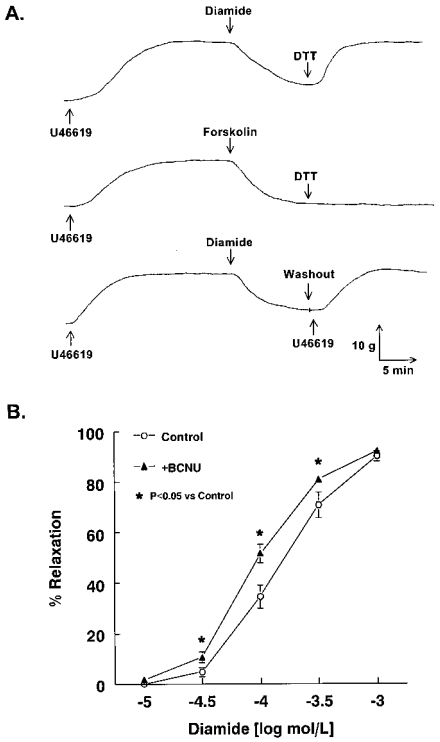


Figure 1. Evidence for reversibility and role of thiol redox in relaxation of BCAs to diamide. A, Typical experimental records of 0.1 μmol/L U46619–precontracted BCAs showing effect of thiol reductant DTT (1 mmol/L) on relaxation to 0.3 mmol/L diamide (top) or 1 μmol/L forskolin (middle) and effect of washout of diamide (bottom). B, Relaxation of BCAs precontracted with U46619 to similar levels of force in absence and presence of inhibition of GSH reductase by pretreatment of BCAs for 30 minutes with 0.1 mmol/L BCNU to increasing cumulative concentrations of diamide.

An Inhibitor of GSH Reductase Enhances BCA Relaxation to Diamide

Inhibition of GSH reductase[23] by a 30-minute pretreatment with 0.1 mmol/L BCNU followed by washout was used to examine whether preventing the reduction of oxidized GSH enhanced the response to the thiol oxidant diamide. The conditions used for the BCNU treatment were optimized in preliminary experiments to minimize its previously reported inhibitory effects on force generation and relaxation to NO.[22] Although BCNU pretreatment did not significantly depress the contraction caused by the subsequent addition of 0.1 μmol/L U46619, the dose of U46619 was increased to 0.3 μmol/L in BCNU-pretreated vessels, which showed a slow development of force. Under these conditions, as shown in Figure 1B, BCNU pretreatment enhanced relaxation to increasing cumulative concentrations of diamide. Under similar conditions, BCNU pretreatment did not significantly alter the relaxation responses to increasing cumulative concentrations of the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP; see panel C of Figure I, which can be accessed online at http://atvb.ahajournals.org) or forskolin, a stimulator of cAMP production (see panel D of online Figure I).

Effects of Inhibitors of sGC on BCA Relaxation to Diamide

Pretreatment with inhibitors of the activation of sGC, including 10 μmol/L ODQ, methylene blue, and LY83583, was

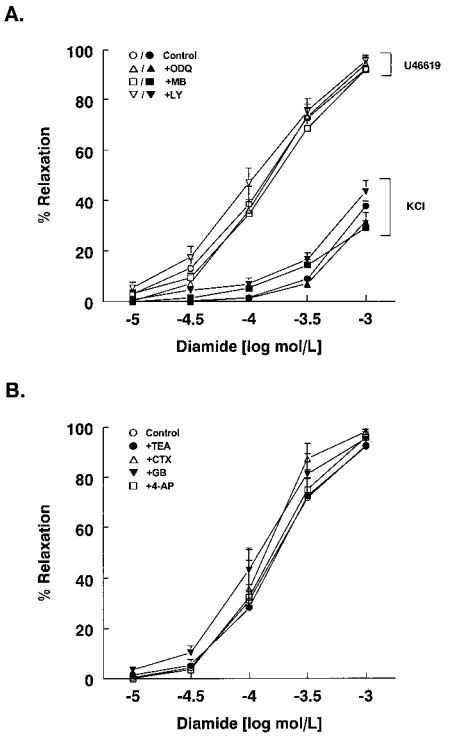


Figure 2. Evidence against role for stimulation of sGC and opening of K^+ channels in mechanism of BCA relaxation to diamide. A, Effects of inhibitors of sGC on diamide-elicited relaxation of BCAs precontracted with 0.1 μmol/L U46619 (n=6 to 8) or 30 mmol/L KCl (n=8). ODQ indicates 10 μmol/L ODQ; MB, 10 μmol/L methylene blue; and LY, 10 μmol/L LY83583. B, Effects of K^+ channel blockers on diamide-elicited relaxation of BCAs precontracted with U46619 (n=4 to 6). TEA indicates 10 mmol/L TEA; CTX, 50 nmol/L CTX; GB, 10 μmol/L glibenclamide; and 4-AP, 1 mmol/L 4-aminopyridine.

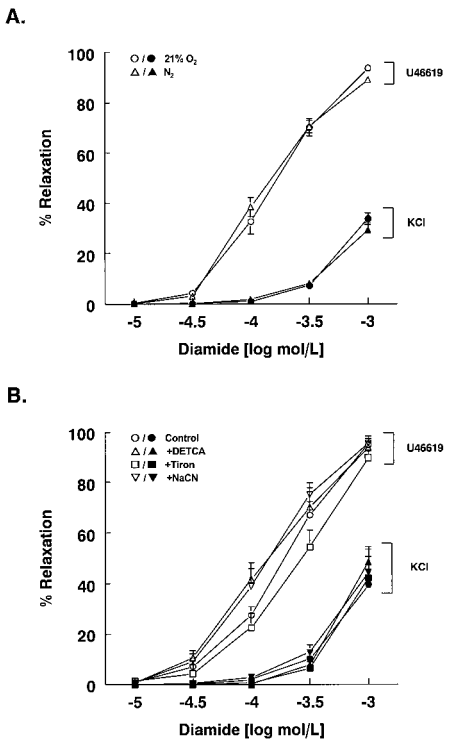


Figure 3. Evidence against role for O_2-dependent processes, superoxide and redox-regulated tyrosine kinases, in the mechanism of BCA relaxation to diamide. A, Relaxation to diamide under normoxic (21% O_2) or severely hypoxic (N_2) conditions in BCAs precontracted with U46619 (n=6) or KCl (n=18). B, Effects of probes that modulate levels of superoxide on diamide-elicited relaxation in BCAs precontracted with U46619 (n=6) or KCl (n=4 to 8). DETCA indicates pretreatment with 10 mmol/L DETCA; Tiron, 10 mmol/L Tiron; and NaCN, 1 mmol/L NaCN.

used to examine their potential effects on BCA relaxation to diamide. BCAs were initially precontracted with either 0.1 μmol/L U46619 or 30 mmol/L KCl in the absence or presence of the inhibitors of sGC activation, and then the relaxant effects of diamide (10 μmol/L to 1 mmol/L) were examined. As shown in Figure 2A, none of these inhibitors altered relaxation to diamide in BCAs precontracted with either U46619 or KCl. The data in Figure 2A also indicate that relaxation responses to each dose of diamide were markedly reduced ($P < 0.05$) in BCAs precontracted with 30 mmol/L KCl compared with BCAs precontracted with 0.1 μmol/L U46619.

Effects of K^+ Channel Blockers on BCA Relaxation to Diamide

K^+ channel blockers for channels including the Ca^{2+}-dependent K^+ channels (10 mmol/L TEA and 50 nmol/L CTX), ATP-dependent K^+ channels (10 μmol/L glibenclamide), and voltage-gated K^+ channels (1 mmol/L 4-aminopyridine)[24] were used to examine their potential effects on BCA relaxation to diamide. BCAs were initially precontracted with 0.1 μmol/L U46619 in the absence or presence of the K^+ channel blockers, and then the relaxant effects of diamide (10 μmol/L to 1 mmol/L) were examined. As shown in Figure 2B, pretreatment with these blockers did not alter the BCA relaxation to diamide in U46619-precontracted BCAs. The combined presence of CTX and glibenclamide also did not alter the relaxation to diamide (n=4, not shown).

Effects of Probes for O_2 Metabolism on BCA Relaxation to Diamide

To examine whether O_2 tension affects diamide-elicited relaxation, experiments were performed in either normoxic or severely hypoxic conditions. BCAs were initially precontracted with either 0.1 μmol/L U46619 or 30 mmol/L KCl in the absence or presence of a severely hypoxic atmosphere (95% N_2/5% CO_2, PO_2 8 to 10 mm Hg). Hypoxic conditions did not affect the relaxation to diamide in BCAs precontracted with either U46619 or KCl (see Figure 3A). The effects of altering BCA levels of superoxide on the response to diamide were examined to determine whether the levels of this species influenced relaxation to this agent. Superoxide levels were increased by inhibition of CuZn superoxide dismutase either by using a 30-minute pretreatment with 10 mmol/L diethyldithiocarbamic acid (DETCA), followed by washout before contraction,[15] or by contracting BCAs with either 0.1 μmol/L U46619 or 30 mmol/L KCl in the presence of 1 mmol/L NaCN.[25] Superoxide levels were lowered by contracting BCAs with either 0.1 μmol/L U46619 or 30 mmol/L KCl in the presence of an intracellular scavenger of superoxide that promotes H_2O_2 formation (10 mmol/L 4,5-dihydroxy-1,3-benzenedisulfonic acid [Tiron]).[16] As shown by the data in Figure 3B, these methods of altering BCA levels of superoxide did not alter the relaxation elicited by subsequent exposure of the BCAs to increasing cumulative concentrations of diamide (10 μmol/L to 1 mmol/L) in

BCAs precontracted with either U46619 or KCl. None of the probes examined had a significant effect on contraction to U46619, whereas N_2 and DETCA decreased ($P < 0.05$) contraction to KCl by 26% and 35%, respectively.

Effects of Inhibition of Tyrosine Kinases on BCA Relaxation to Diamide

The effects of inhibiting tyrosine kinase activity in BCAs with 10 μmol/L genistein on the relaxation to diamide was examined to determine whether any of the thiol redox–sensitive processes that influence signaling through tyrosine phosphorylation participate in this response. BCAs were initially precontracted with either 0.1 μmol/L U46619 or 30 mmol/L KCl in the absence or presence of genistein, and then the relaxant effects of diamide (10 μmol/L to 1 mmol/L) were examined. This agent did not significantly alter the response to diamide in BCAs precontracted with either U46619 or KCl (panel C of Figure II, which can be accessed online at http://atvb.ahajournals.org).

Effects of Different Types of Contractile Stimuli on BCA Relaxation to Diamide

To examine whether the sensitivity of BCA relaxation to diamide is dependent on the signaling mechanisms involved in the stimuli used for contraction, vessels were precontracted to similar levels of force with either 0.1 μmol/L U46619, 1 μmol/L Bay K8644 (L-type Ca^{2+} channel opener), or 10 μmol/L PDBu (protein kinase C activator), and then the concentration dependence of the diamide-elicited relaxation was compared. Bay K8644 was applied in the presence of 14.7 mmol/L KCl (a concentration of KCl that did not affect vascular tone), because Bay K8644 requires a subthreshold depolarization to cause contraction.[26] As shown in Figure 4A, diamide-elicited relaxation was almost eliminated when vessels were precontracted with PDBu. Although there was a right shift in the concentration-response curve when vessels were precontracted with Bay K8644 compared with U46619, 1 mmol/L diamide caused almost full relaxation of BCAs precontracted with either agent. To elucidate whether PDBu-elicited contraction is modulated by the presence of extracellular Ca^{2+}, vessels were contracted in Ca^{2+} -free solution containing 1 mmol/L EGTA, and then the maximal stable contraction was compared with that in normal Krebs' solution. PDBu (10 μmol/L) caused substantial contraction even in Ca^{2+}-free solution, and this contraction was only 29% (n=15) less than that in Ca^{2+}-containing solution, indicating that PDBu-elicited contraction is largely independent of extracellular Ca^{2+}.

To assess whether other relaxants cause vascular relaxation when vessels are precontracted with PDBu, the NO donor SNAP or the cAMP stimulant forskolin were applied to BCAs precontracted with U46619, Bay K8644, or PDBu. Although each contractile agent showed different sensitivity to vasorelaxant agents, SNAP (Figure 4B) and forskolin (see panel C of Figure III, which can be accessed online at http://atvb.ahajournals.org) caused substantial relaxation of PDBu-precontracted BCAs.

Effects of Diamide on Contraction to Different Types of Stimuli Elicited in Ca^{2+}-Free Solution Followed by Addition of $CaCl_2$

In this series of experiments, vessels were first contracted with 30 mmol/L KCl in normal Krebs' solution, followed by washout, and subsequent contractile responses were expressed as a percentage of the initial response to 30 mmol/L KCl. BCAs were then placed in Ca^{2+}-free Krebs' solution containing 0.1 mmol/L EGTA, and then the contractile effects of 10 μmol/L 5-hydroxytryptamine (5-HT), 30 mmol/L KCl, or 1 μmol/L Bay K8644 (14.7 mmol/L KCl) were examined in the absence or presence of 1 mmol/L diamide, followed by addition of 1.5 mmol/L $CaCl_2$ to detect contractile responses originating from the initial release of intracellular Ca^{2+} and the subsequent influx of extracellular Ca^{2+}, respectively. Diamide was added 10 minutes before the application of contractile agents, and the initial response was observed for 5 to 10 minutes before the addition of $CaCl_2$.

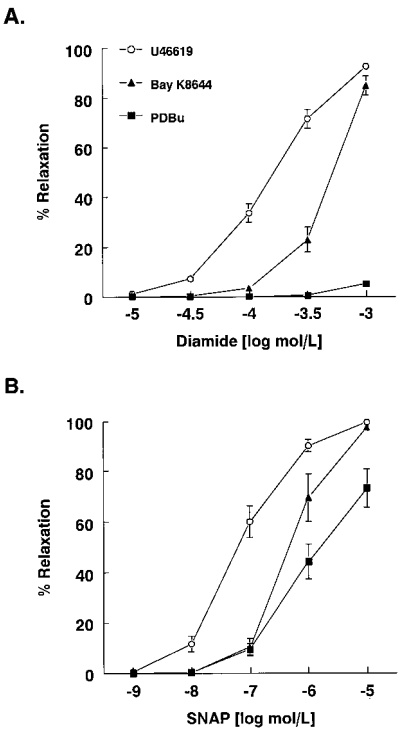
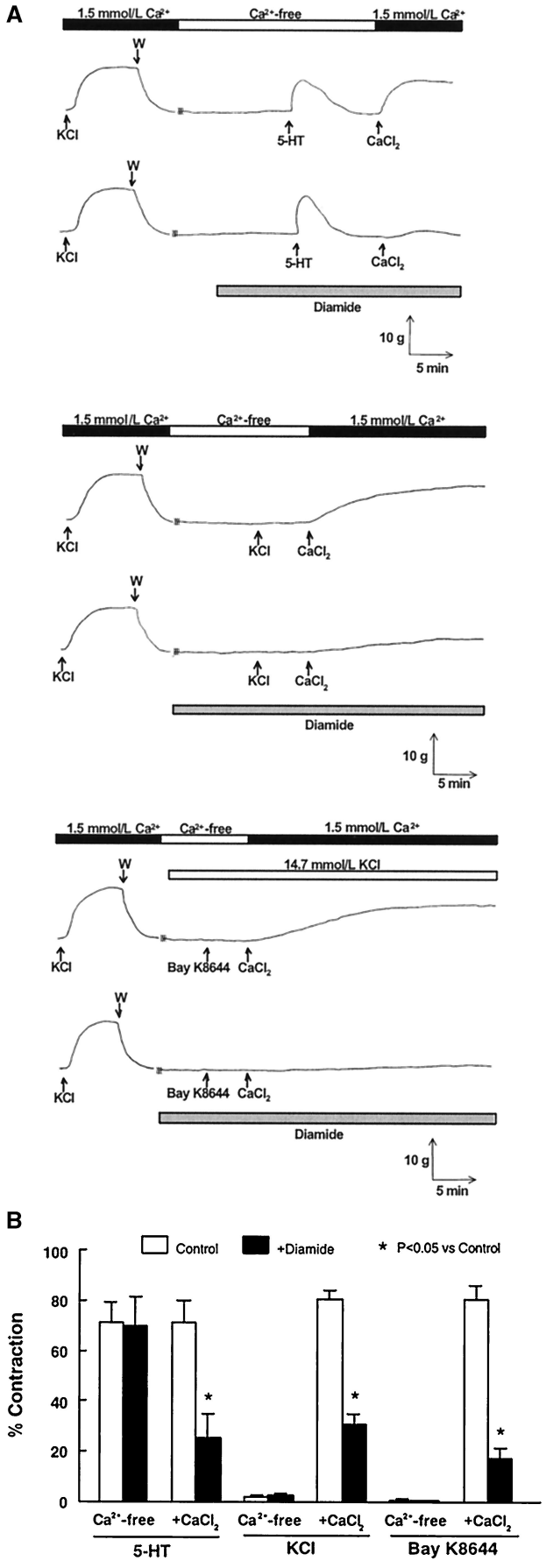


Figure 4. Effects of contractile agents on relaxation of BCAs to diamide. A, Relaxation to diamide in BCAs precontracted with 0.1 μmol/L U46619 (n=11), 1 μmol/L Bay K8644 (n=9), or 10 μmol/L PDBu (n=21). B, Relaxation to SNAP in BCAs precontracted with U46619 (n=9), Bay K8644 (n=10), or PDBu (n=21).

5-Hydroxytryptamine

Figure 5A (top) shows a typical BCA response to 10 μmol/L 5-HT in Ca^{2+}-free solution and the subsequent contraction elicited by the addition of 1.5 mmol/L $CaCl_2$ either in the absence or presence of 1 mmol/L diamide. In the absence of diamide, 5-HT caused rapid transient contraction, followed by sustained tonic contraction on addition of $CaCl_2$. In the presence of 1 mmol/L diamide, the initial transient contraction was not significantly affected, but subsequent contraction elicited by $CaCl_2$ was markedly inhibited. These responses are summarized in Figure 5B. In some separate experiments in which 5-HT was washed out after the initial transient contraction, no apparent contraction was observed after adding $CaCl_2$, indicating that capacitative Ca^{2+} entry[27] had a minimum role, if any, in the contraction elicited by 5-HT under these experimental conditions (n=4, not shown).



Potassium Chloride

Figure 5A (middle) shows a typical BCA response to 30 mmol/L KCl in Ca^{2+}-free solution and the subsequent contraction elicited by the addition of 1.5 mmol/L $CaCl_2$ in the absence or presence of 1 mmol/L diamide. No significant contraction was observed in Ca^{2+}-free solution in the absence or presence of 1 mmol/L diamide. Subsequent addition of 1.5 mmol/L $CaCl_2$ caused a sustained contraction in the absence of diamide; however, this sustained contraction was markedly inhibited by the presence of 1 mmol/L diamide. These responses are summarized in Figure 5B. In some separate experiments in which KCl was washed out after 5 minutes, no apparent contraction was observed after adding $CaCl_2$, indicating that capacitative Ca^{2+} entry had a minimum role, if any, in the contraction elicited by KCl under these experimental conditions (n=4, not shown).

Bay K8644

Figure 5A (bottom) shows a typical BCA response to an L-type Ca^{2+} channel opener, 1 μmol/L Bay K8644, in Ca^{2+}-free solution and the subsequent contraction elicited by the addition of 1.5 mmol/L $CaCl_2$ in the absence or presence of 1 mmol/L diamide. Similar to the responses to 30 mmol/L KCl, Bay K8644 did not cause any significant contraction in Ca^{2+}-free solution, and the subsequent addition of 1.5 mmol/L $CaCl_2$ caused a sustained contraction in the absence of diamide; this sustained contraction was markedly inhibited by the presence of 1 mmol/L diamide. These responses are summarized in Figure 5B.

Effects of L-Type Ca^{2+} Channel Blockers on BCA Relaxation to Diamide

The effects of L-type Ca^{2+} channel blockers nifedipine and diltiazem on the response to diamide were examined to determine whether these channels contributed to its relaxant actions. In initial experiments, these agents were observed to cause a similar degree of inhibition of contraction to 0.1 μmol/L U46619 or 30 mmol/L KCl at each dose examined. The inhibition of contraction of 53.5±2.0% by 1 μmol/L nifedipine (n=4) and 72.0±4.2% by 10 μmol/L diltiazem (n=4) could be reversed by increasing the concentration of U46619 from 0.1 to 0.3 to 1.0 μmol/L. In contrast, it was difficult to obtain similar levels of force in the absence or presence of nifedipine or diltiazem through increasing the concentration of KCl. As shown in Figure 6A, 1 μmol/L nifedipine and 10 μmol/L diltiazem inhibited the relaxation to increasing cumulative concentrations of diamide. Under similar conditions, the presence of nifedipine or diltiazem had minimal effects on relaxation responses to increasing cumulative concentrations of SNAP (Figure 6B) or forskolin (see panel C of Figure IV, which can be accessed online at http://atvb.ahajournals.org).

Figure 5. Evidence that inhibition of Ca^{2+} influx contributes to mechanism of BCA relaxation to diamide. Shown are contractile responses of BCAs in Ca^{2+}-free (0.1 mmol/L EGTA) solution, followed by addition of 1.5 mmol/L $CaCl_2$ in absence or presence of 1 mmol/L diamide. A, Typical experimental responses to 10 μmol/L 5-HT (top), 30 mmol/L KCl (middle), and 1 μmol/L Bay K8644 (14.7 mmol/L KCl, bottom) in absence (upper trace) or presence (lower trace) of diamide. B, Summary data for 5-HT (n=12), KCl (n=12), and Bay K8644 (n=17).

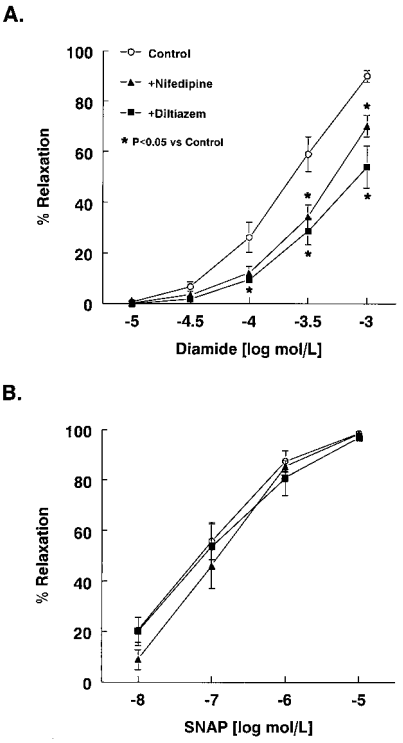


Figure 6. Effects of L-type Ca^{2+} channel blockers on relaxation of BCA to diamide. Shown is relaxation of BCAs precontracted with U46619 to similar levels of force in absence or presence of 1 μmol/L nifedipine and 10 μmol/L diltiazem to increasing cumulative concentrations of diamide (n=7 to 8, A) and SNAP (n=5 to 7, B).

Discussion

In the present study, the thiol oxidant diamide was observed to cause a reversible concentration-dependent relaxation of endothelium-removed BCAs, which appeared to be mediated through alterations in a thiol redox process, inasmuch as the response was enhanced by an inhibitor of GSH reductase, and it was reversed by the thiol reductant DTT or by removal of the diamide. Previous studies on a variety of cardiovascular tissue and cellular preparations (eg, see References 20 to 22) have demonstrated that the concentrations of diamide observed to cause coronary arterial relaxation in the present study consistently cause severe degrees of reversible GSH oxidation and S-thiolation of proteins. The actions of probes used in the present study suggest that the relaxing mechanism activated by diamide does not seem to involve the stimulation of sGC or tyrosine kinases, the opening of K^+ channels, or a process involving O_2 metabolism or the modulation of reactive O_2 species. Under the conditions of the present study, diamide appears to function primarily through inhibiting a mechanism of Ca^{2+} influx, and evidence of its promotion of relaxation through influencing the signaling mechanisms associated with the 5-HT receptor–mediated release of Ca^{2+} from intracellular organelles or contractile mechanisms regulated through the stimulation of protein kinase C could not be detected.

Thiol redox has multiple ways of potentially interacting with systems that regulate the activity of sGC. Because the metabolism of H_2O_2 by GSH peroxidase appears to function as a modulator of the stimulation of sGC by peroxide metabolism by catalase in BCAs,[17] diamide could cause relaxation by increasing the intracellular levels of H_2O_2 and its metabolism by catalase. However, this mechanism does not appear to mediate the relaxation to diamide, inasmuch as it has been shown in the present study that inhibitors of sGC stimulation do not alter relaxation to this agent. In a previous study, it has also been observed that the inactivation of catalase does not alter the relaxation of endothelium-removed bovine coronary arteries to diamide.[17] In addition, the catalytic activity of sGC appears to be inactivated by disulfide formation or S-thiolation through reactions with disulfides, including the oxidized form of GSH,[6] and this process may function as an endogenous mechanism of inhibiting the vascular form of sGC.[7] Thus, the stimulation of sGC does not appear to be involved in the relaxation to diamide.

There is substantial evidence that many of the different forms of K^+ channels are potentially regulated by thiol redox processes.[4,28] In the present study, it was observed that agents that inhibit most of the known forms of K^+ channels did not alter the response to diamide. Because hypoxia did not alter relaxation to diamide, it appears that processes associated with the P_{O_2}-dependent regulation of K^+ channels[4,5] did not contribute to preventing the detection of a role for K^+ channels in the actions of this thiol oxidant. Thus, a relaxing mechanism activated by hyperpolarization as a result of the opening of plasma membrane K^+ channels does not seem to participate in the mechanism of relaxation to diamide.

The absence of effects of other probes used in the present study that altered O_2 metabolism on relaxation to diamide were consistent with the absence of a role for several additional mechanisms. Because modulating superoxide levels, NaCN, and severe hypoxia did not alter the response to diamide, it appears that relaxation to this agent does not involve alterations in a signaling mechanism dependent on the formation of reactive O_2 species (eg, H_2O_2) or other processes controlled by P_{O_2}, such as energy metabolism. Because superoxide-derived oxidants and thiol redox signaling appear to activate multiple genistein-inhibitable tyrosine kinase–dependent signaling mechanisms, including processes dependent on activation of $p21^{ras}$, mitogen-activated protein kinases, and phospholipases,[9–11] these processes may also not be involved in relaxation to diamide.

Various approaches were investigated to probe the role of systems that control the levels of intracellular Ca^{2+} in BCAs because systems such as sarcoplasmic reticulum Ca^{2+} uptake is thought to be regulated by oxidant mechanisms through changes in thiol redox.[13,14] The absence of a relaxation to diamide in BCAs contracted with PDBu, an activator of protein kinase C, suggested that a key aspect of the mechanism of action of diamide could involve either a process that is inhibited by protein kinase C or a system that controls the levels of intracellular Ca^{2+}. This latter hypothesis is based on the observation that contraction to PDBu is largely independent of the presence of extracellular Ca^{2+} under the conditions examined in the present study. It is important to note that PDBu does not prevent BCA relaxation in a nonspecific manner, because SNAP and forskolin caused substantial relaxation in BCAs contracted with PDBu. Because the initial contraction to 5-HT in the absence of extracellular Ca^{2+} was not altered by diamide, this thiol oxidant does not appear to be influencing the processes involved in the release of sarcoplasmic reticulum Ca^{2+} by the 5-HT receptor–regulated mechanism. The absence of an inhibition of force generation by the protein kinase C activator PDBu or the initial contrac-

tion to 5-HT in the absence of Ca^{2+} is also consistent with diamide not directly altering the function of contractile proteins involved in the generation of force under the conditions of these experiments. However, further increases in thiol oxidation could influence these thiol-dependent signaling systems. Observation of a marked inhibition of contraction caused by the readdition of Ca^{2+} in the presence of 5-HT or agents that activate voltage-dependent Ca^{2+} channels implicates a role for inhibition of sarcolemmal Ca^{2+} influx in the mechanism of relaxation to diamide.

The L-type Ca^{2+} channel is one of the best identified Ca^{2+} channels in smooth muscle, and this channel may be the primary channel that mediates Ca^{2+} influx associated with receptor and voltage-dependent contraction in smooth muscle.[27] Because L-type Ca^{2+} channel blockers selectively attenuated the response to diamide, these channels appear to contribute to its mechanism of relaxation. The observation that L-type Ca^{2+} channel blockers had a similar potency in relaxing BCAs contracted with KCl and U46619, compared with the markedly reduced potency of diamide on arteries contracted with KCl, suggests that diamide may also be simultaneously activating an additional mechanism that could potentially function through enhancing the contractile actions of KCl. Interestingly, studies on the cloned smooth muscle L-type Ca^{2+} channel have identified a key role for a thiol redox process in controlling its activity, in which oxidation closes the channel.[29] Although diamide could be functioning in a manner similar to that of L-type Ca^{2+} channel blockers, the observation that relaxation to diamide is enhanced in the presence of inhibition of GSH reductase is consistent with a mechanism controlled by intracellular thiol redox. Thus, diamide appears to function through activating a thiol oxidation mechanism that inhibits L-type Ca^{2+} channels.

The present study has identified a potentially novel vasodilator mechanism in BCAs that appears to be mediated through closure of a plasma membrane channel that seems to be the L-type Ca^{2+} channel by a thiol oxidation mechanism. Because removal of the diamide caused a rapid reversal of this relaxation, enzymes that normally control cellular thiol redox processes, including GSH and thioredoxin reductases,[12] are likely to participate in reversal of the alterations caused by diamide. Although it is possible that some of the other thiol redox–related signaling mechanisms examined in the present study are altered by diamide, it appears that closure of a plasma membrane Ca^{2+} channel is the dominant relaxing mechanism activated in BCAs under the conditions examined. The coronary vasodilator mechanism investigated in the present study may be of importance in situations associated with thiol oxidation caused by oxidant and/or nitrosative stress, such as cardiac ischemia/reperfusion and inflammation.

Acknowledgments

This work was supported by US Public Health Service Grants HL-31069 and HL-43023. T. Iesaki was partly supported by a Research Fellowship from the Uehara Memorial Foundation.

References

1. Wolin MS. Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol*. 2000;20:1430–1442.
2. Kosower NS, Kosower EM. Diamide: an oxidant probe for thiols. *Methods Enzymol*. 1995;251:123–133.
3. Weir EK, Will JA, Lundquist LJ, Eaton JW, Chesler E. Diamide inhibits pulmonary vasoconstriction induced by hypoxia and prostaglandin $F_{2\alpha}$. *Proc Soc Exp Biol Med*. 1983;173:96–103.
4. Weir EK, Archer SL. The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. *FASEB J*. 1995;9:183–189.
5. Archer SL, Will JA, Weir EK. Redox status in the control of pulmonary vascular tone. *Herz*. 1986;11:127–141.
6. Brandwein HJ, Lewicki JA, Murad F. Reversible inactivation of guanylate cyclase by mixed disulfide formation. *J Biol Chem*. 1981;256:2958–2962.
7. Gupte S, Rupawalla BA, Phillibert D, Wolin MS. NADPH and heme redox modulate pulmonary artery relaxation and guanylate cyclase activation by NO. *Am J Physiol*. 1999;277:L1124–L1132.
8. Wolin MS, Burke-Wolin TM, Mohazzab-H KM. Roles for NAD(P)H oxidases and reactive oxygen species in vascular oxygen sensing mechanisms. *Respir Physiol*. 1999;115:229–238.
9. Rao GN. Hydrogen peroxide induces complex formation of SHC-Grb2-SOS with receptor tyrosine kinase and activates ras and extracellular signal regulated protein kinases group of mitogen activated protein kinases. *Oncogene*. 1996;272:713–719.
10. Natarajan V, Scribner WN, Al-Hassani M, Vepa S. Reactive oxygen species signaling through regulation of protein tyrosine phosphorylation in endothelial cells. *Environ Health Perspect*. 1998;106:1205–1212.
11. Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res*. 1999;85:753–766.
12. Thomas JA, Poland B, Honzatko R. Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. *Arch Biochem Biophys*. 1995;319:1–9.
13. Grover AK, Samson SE, Fomin VP, Werstiuk ES. Effects of peroxide and superoxide on coronary artery: angiotensin II and sarcoplasmic reticulum Ca^{2+} pump. *Am J Physiol*. 1995;269:C546–C553.
14. Suzuki YJ, Ford GD. Redox regulation of signal transduction in cardiac and smooth muscle. *J Mol Cell Cardiol*. 1999;31:345–353.
15. Omar HA, Cherry PD, Mortelliti MP, Burke-Wolin T, Wolin MS. Inhibition of coronary artery superoxide dismutase attenuates endothelium-dependent and independent nitrovasodilator relaxation. *Circ Res*. 1991;69:601–608.
16. Mohazzab-H KM, Fayngersh RP, Kaminski PM, Wolin MS. Oxygen-elicited responses in calf coronary arteries: role of H_2O_2 production via NADH-derived superoxide. *Am J Physiol*. 1996;270:H1044–H1053.
17. Mohazzab-H KM, Agarwal R, Wolin MS. Influence of glutathione peroxidase on coronary artery responses to alterations in PO_2 and H_2O_2. *Am J Physiol*. 1999;276:H235–H241.
18. Iesaki T, Gupte S, Kaminski PM, Wolin MS. Inhibition of guanylate cyclase stimulation by NO and bovine arterial relaxation to peroxynitrite and H_2O_2. *Am J Physiol*. 1999;277:H978–H985.
19. Iesaki T, Gupte S, Wolin MS. A flavoprotein mechanism appears to prevent an oxygen-dependent inhibition of cGMP-associated nitric oxide–elicited relaxation of bovine coronary arteries. *Circ Res*. 1999;85:1027–1031.
20. Collison MW, Thomas JA. S-Thiolation of cytoplasmic cardiac creatine kinase in heart cells treated with diamide. *Biochim Biophys Acta*. 1987;928:121–129.
21. Kokura S, Wolf RE, Yoshikawa T, Granger DN, Aw T. Molecular mechanism of neutrophil–endothelial cell adhesion induced by redox balance. *Circ Res*. 1999;84:516–524.
22. Adachi T, Cohen RA. Decreased aortic glutathione levels may contribute to impaired nitric oxide-induced relaxation in hypercholesterolaemia. *Br J Pharmacol*. 2000;129:1014–1020.
23. Becker K, Schirmer RH. 1,3-Bis(2-choloroethyl)-1-nitrosourea as thiol-carbamoylating agent in biological systems. *Methods Enzymol*. 1995;251:173–188.
24. Reeve HL, Weir EK, Archer SL, Cornfield DN. A maturational shift in pulmonary K^+ channels, from Ca^{2+} sensitive to voltage dependent. *Am J Physiol*. 1998;275:L1019–L1025.
25. Fridovich I. Superoxide dismutases. *Adv Enzymol*. 1974;41:35–97.
26. Schramm M, Thomas G, Towart R, Franckowiak G. Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature*. 1983;303:535–537.
27. Karaki H, Ozaki H, Hori M, Mitsui-Saito M, Amano K-I, Harada K-I, Miyamoto S, Nakazawa H, Won K-J, Sato K. Calcium movements, distribution, and functions in smooth muscle. *Pharmacol Rev*. 1997;49:157–230.
28. Duprat F, Guillemare E, Romey G, Fink M, Lesage F, Lazdunski M. Susceptibility of cloned K^+ channels to reactive oxygen species. *Proc Natl Acad Sci U S A*. 1995;92:11796–11800.
29. Chiamvimonvat N, O'Rourke B, Kamp TJ, Kallen RG, Hofmann F, Flocherzi V, Marban E. Functional consequences of sulfhydryl modification in the pore-forming subunits of cardiovascular Ca^{2+} and Na^+ channels. *Circ Res*. 1996;78:723–734.

Supplement to ATVB Ms 794

Figure Legends

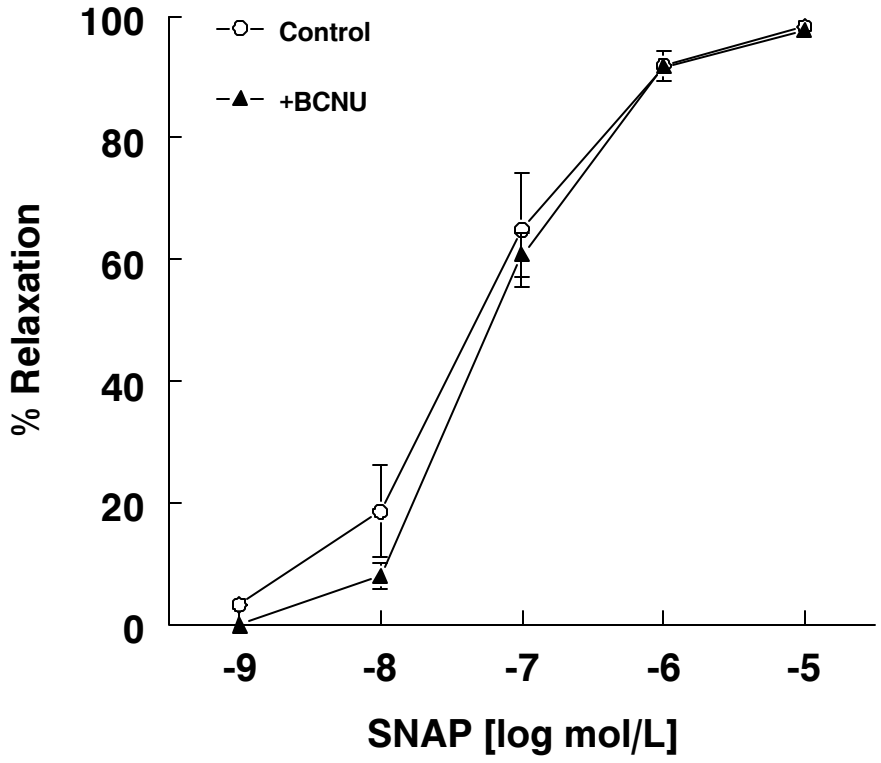
Fig. I. Supplement to Fig. 1: Evidence for the reversibility and role of thiol redox in relaxation of BCA to diamide. Panel C and D: Relaxation of BCA precontracted with U46619 to similar levels of force in the absence and presence of inhibition of GSH reductase by pretreatment of BCA for 30 minutes with 0.1 mmol/L BCNU to increasing cumulative concentrations of SNAP (Panel C, n=4) and forskolin (Panel D, n=7).

Fig. II. Supplement to Fig. 3: Evidence against a role for the O_2-dependent processes, superoxide and redox-regulated tyrosine kinases in the mechanism of BCA relaxation to diamide. Panel C: Effects of an inhibitor of tyrosine kinases (10 µmol/L genistein = GS) on diamide-elicited relaxation of BCA precontracted with U46619 (n=7) or KCl (n=11).

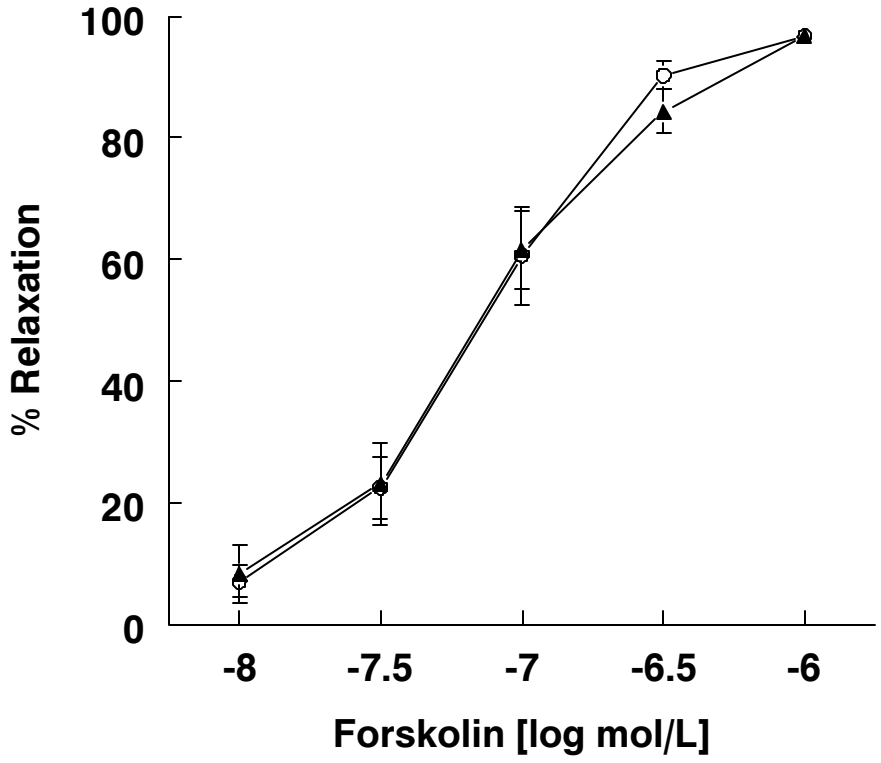
Fig. III. Supplement to Fig. 4: Effects of contractile agents on the relaxation of BCA to diamide. Panel C: Relaxation to forskolin in BCA precontracted with either 0.1 µmol/L U46619 (n=9), 1 µmol/L Bay K8644 (n=9) or 10 µmol/L PDBu (n=13).

Fig. IV. Supplement to Fig. 6: Effects of L-type calcium channel blockers on the relaxation of BCA to diamide. Panel C: Relaxation of BCA precontracted with U46619 to similar levels of force in the absence or presence of 1 µmol/L nifedipine and 10 µmol/L diltiazem to increasing cumulative concentrations of forskolin (n=6).

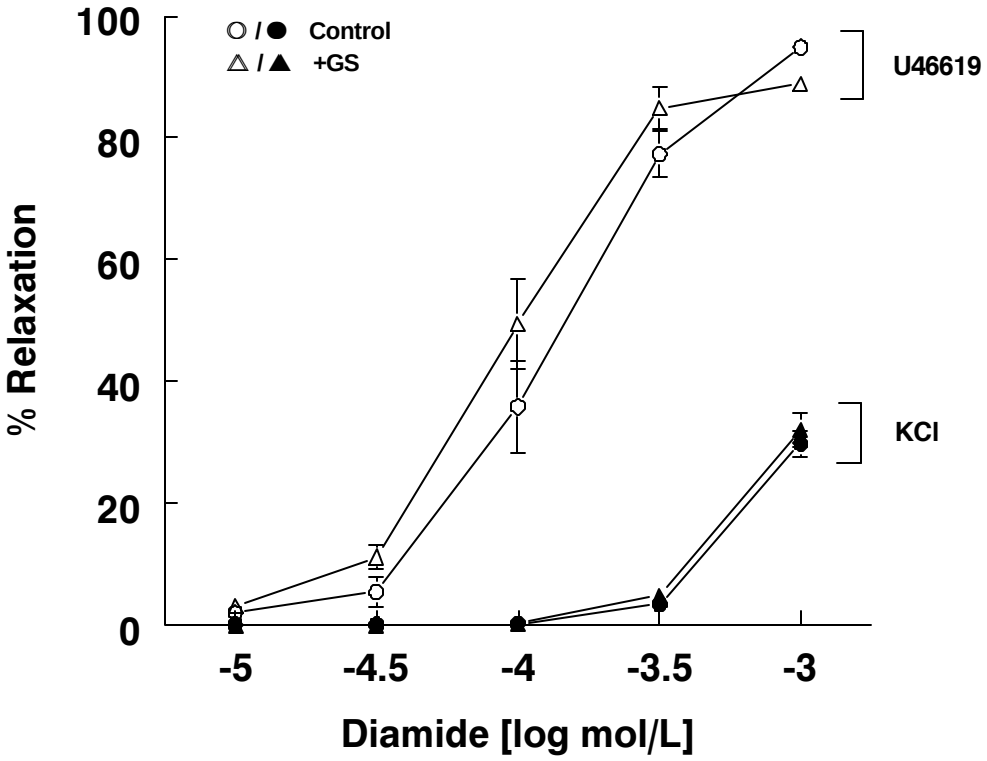
C.


Control
+BCNU
% Relaxation
100
80
60
40
20
0
-9
-8
-7
-6
-5
SNAP [log mol/L]

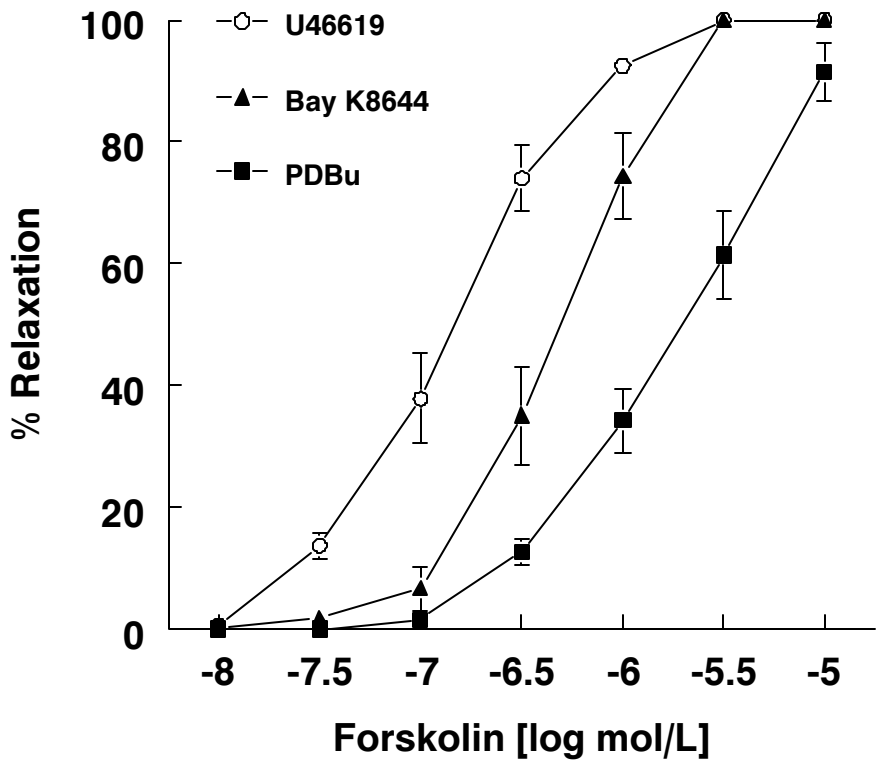
D.


% Relaxation
100
80
60
40
20
0
-8
-7.5
-7
-6.5
-6
Forskolin [log mol/L]

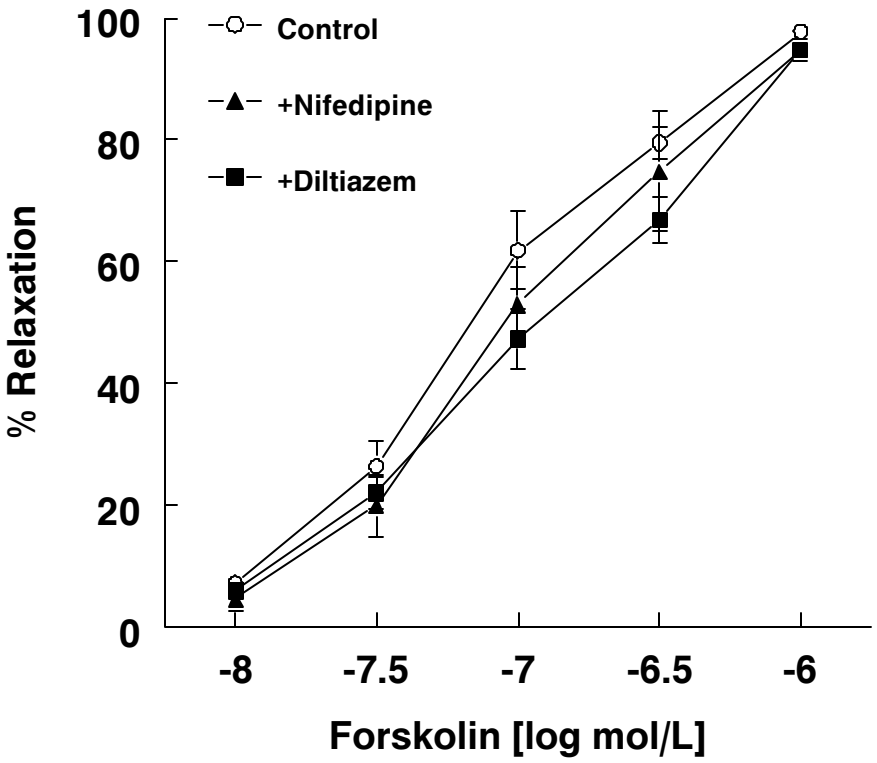
C.


100
80
60
40
20
0
% Relaxation
○ / ● Control
△ / ▲ +GS
U46619
KCl
-5
-4.5
-4
-3.5
-3
Diamide [log mol/L]

C.


100
80
60
40
20
0
% Relaxation
U46619
Bay K8644
PDBu
-8
-7.5
-7
-6.5
-6
-5.5
-5
Forskolin [log mol/L]

C.


100
80
60
40
20
0
% Relaxation
Control
+Nifedipine
+Diltiazem
-8
-7.5
-7
-6.5
-6
Forskolin [log mol/L]

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Thiol Oxidation Activates a Novel Redox-Regulated Coronary Vasodilator Mechanism Involving Inhibition of Ca^{2+} Influx

Takafumi Iesaki and Michael S. Wolin

Arterioscler Thromb Vasc Biol. 2000;20:2359-2365
doi: 10.1161/01.ATV.20.11.2359
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636