Activation of Mitochondrial ATP-Dependent Potassium Channels by Nitric Oxide

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Background—Nitric oxide (NO) has been implicated as a mediator of "second-window" ischemic preconditioning, and mitochondrial ATP-dependent K^+ (mitoK_{ATP}) channels are the likely effectors. The links between NO and mitoK_{ATP} channels are unknown.

Methods and Results—We measured mitochondrial redox potential as an index of mitoK_{ATP} channel opening in rabbit ventricular myocytes. The NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP, 0.1 to 1 mmol/L) oxidized the mitochondrial matrix dose-dependently without activating sarcolemmal K_{ATP} channels. SNAP-induced oxidation was blocked by the selective mitoK_{ATP} channel blocker 5-hydroxydecanoate and by the NO scavenger 2-(4-carboxyphenyl)-4,4′,5,5′-tetramethylimidazole-1-oxyl-3-oxide. SNAP-induced mitochondrial oxidation was detectable either by photomultiplier tube recordings of flavoprotein fluorescence or by confocal imaging. SNAP also enhanced the oxidative effects of diazoxide when both agents were applied together. Exposure to 1 mmol/L 8Br-cGMP failed to mimic the effects of SNAP.

Conclusions—NO directly activates mitoK_{ATP} channels and potentiates the ability of diazoxide to open these channels. These results provide novel mechanistic links between NO-induced cardioprotection and mitoK_{ATP} channels. **(*Circulation*. 2000;101:439-445.)**

Key Words: ischemic preconditioning ■ nitric oxide ■ myocytes ■ mitochondria

Nitric oxide is a key signaling molecule that figures prominently in ischemic preconditioning. NO is generated during the oxidation of L-arginine to citrulline, not only in the cytosol but also in mitochondria.[1–3] During ischemia, the endogenous production of NO in the heart is increased.[1] It is also common therapeutic practice to administer exogenous nitrates in acute ischemic syndromes, further boosting the concentration of NO. Although NO has been reported to afford cardioprotection from reperfusion injury,[4–6] the role of NO during the early phase of ischemic preconditioning is controversial.[7–10] Conversely, several lines of evidence convincingly implicate NO as a mediator of the late phase (second window) of ischemic preconditioning against infarction and stunning.[11–13] The cardioprotective effects of NO have been explained by several factors, such as microvascular effects,[4] antineutrophil action,[4] induction of stress protein,[14] or modulation of cardiac excitability.[7] Recently, Bernardo et al[15] reported that delayed ischemic preconditioning is inhibited by 5-hydroxydecanoate (5HD) in the rabbit heart. Because 5HD is a selective blocker of mitochondrial ATP-dependent potassium (mitoK_{ATP}) channels in rabbit ventricular cells,[16] the ability of 5HD to abolish second-window protection motivated us to look for possible links between NO and mitoK_{ATP} channels.

Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication 85-23, revised 1985).

Materials

Collagenase (type II) was purchased from Worthington. Diazoxide, 2,4-dinitrophenol (DNP), sodium cyanide (CN), SNAP, and 8-bromo cGMP (8Br-cGMP) were obtained from Sigma Chemical Co. 5HD, pinacidil, and 2-(4-carboxyphenyl)-4,4′,5,5′-tetramethylimidazole-1-oxyl 3-oxide (carboxy-PTIO) were purchased from Research Biochemical International. Diazoxide, SNAP, pinacidil, and carboxy-PTIO were dissolved in DMSO before they were added to experimental solutions. The final concentration of DMSO was <0.1%.

Cell Isolation and Measurement of Mitochondrial Redox State

Rabbit ventricular myocytes were isolated enzymatically from adult rabbit hearts and placed in primary culture as described previously.[16,17] Experiments were performed over the next day. MitoK_{ATP} channel activity was monitored noninvasively by measuring flavoprotein fluorescence as an index of mitochondrial redox state with or without simultaneous whole-cell membrane current recordings (as indicated).[16–18] Cells were superfused with external solution containing (in mmol/L) NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, and HEPES 10 (pH adjusted to 7.4 with NaOH) at room temperature (≈22°C). Endogenous flavoprotein fluorescence was excited for 100 ms every 6 seconds by a xenon arc lamp with a bandpass filter centered at 480 nm. Emitted fluorescence was recorded at 530 nm by a photomul-

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tiplier tube and digitized. The redox signal was averaged during the excitation window and calibrated at the end of each experiment by exposure to DNP, which uncouples respiration from ATP synthesis and induces maximal oxidation. Therefore, the values of flavoprotein fluorescence are expressed as a percentage of the DNP-induced fluorescence. Individual myocytes were observed with a ×40 objective to monitor fluorescence 1 cell at a time.

Confocal Imaging of Flavoprotein Fluorescence

Confocal images were obtained with a Diaphot 300 inverted fluorescence microscope with a PCM-2000 confocal scanning attachment (Nikon, Inc).[17,18] Fluorescence was excited by the 488-nm line of an argon laser, and the emission at 505 to 535 nm was recorded. A time series of images was collected at intervals of 10 seconds, and baseline, diazoxide, SNAP, SNAP+5HD, CN, and DNP images were enhanced by averaging of 7 sequential images having stable mean fluorescence intensities during exposure to each agent. Images were analyzed on a personal computer with the software program Simple32 (Compix, Inc).

Data Analysis

To evaluate the effects of pharmacological agents on flavoprotein fluorescence, the slope of relative change in the fluorescence during drug application was calculated by a least-squares method. The best-fit line is indicated by a dotted line in Figure 1 (A, B, and C), Figure 3 (top), and Figure 4 (A and B). Pooled data are presented as mean±SEM, and the number of cells or experiments is shown as n. Statistical comparison was evaluated by 1-way ANOVA, with a value of $P<0.05$ considered significant.

Electrophysiological Recordings

In some experiments (Figure 3), whole-cell currents and flavoprotein fluorescence were measured simultaneously. The internal pipette solution contained (in mmol/L) potassium glutamate 120, KCl 25, $MgCl_2$ 0.5, K-EGTA 10, HEPES 10, and MgATP 1 (pH adjusted to 7.2 with KOH). Whole-cell currents were elicited every 6 seconds from a holding potential of −80 mV by 2 consecutive steps to −40 mV (for 100 ms) and 0 mV (for 380 ms), and flavoprotein fluorescence was excited during the 100-ms step to −40 mV. To quantify $I_{K,ATP}$, currents at 0 mV were measured 200 ms into the pulse.

Results

Effects of SNAP on mitoK_{ATP} Channels

Figure 1A shows the time course of flavoprotein fluorescence in a cell exposed first to diazoxide, then to SNAP, and finally again to diazoxide. In the first application, diazoxide reversibly oxidized the flavoproteins. Subsequent exposure to SNAP alone gradually increased flavoprotein oxidation with a slope of 0.59%/min. After a 15-minute exposure to SNAP, mitochondrial oxidation persisted even 5 minutes after washout. Although NO has been reported to inhibit respiration,[2,19,20] the present changes are in the opposite direction to

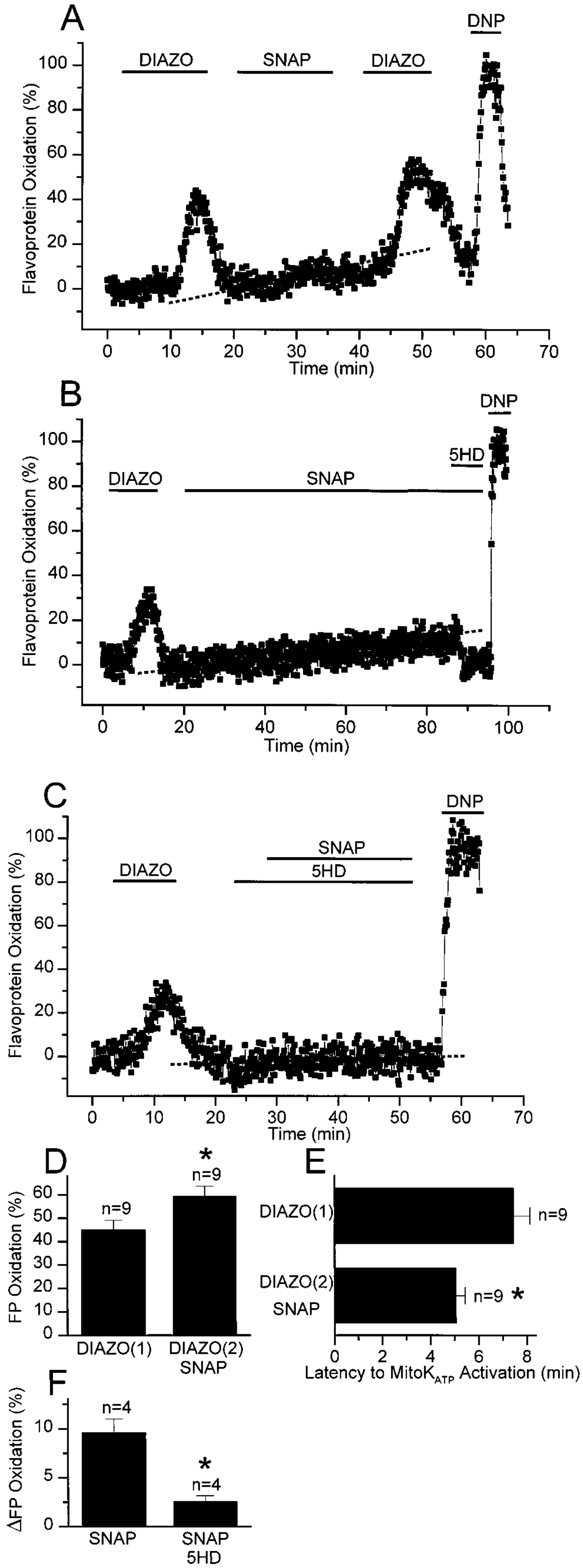

Figure 1. Effects of SNAP on flavoprotein oxidation. A, Time course of flavoprotein fluorescence in a cell exposed twice to diazoxide (DIAZO, 100 μmol/L) with an intervening exposure to SNAP (100 μmol/L). B and C show representative data indicating effects of 5HD (1 mmol/L) on SNAP-induced oxidation. Bars indicate periods when cells were exposed to each drug. Dotted line indicates best fit for changes in flavoprotein fluorescence. D and E, Summarized data for percentage of diazoxide-induced flavoprotein oxidation and latency to mitoK_{ATP} channel activation measured in first [DIAZO(1)] and second exposure after SNAP [DIAZO(2) SNAP]. *$P<0.05$ vs DIAZO(1). F, SNAP-induced flavoprotein oxidation measured in absence (left) and in presence (right) of 1 mmol/L 5HD.

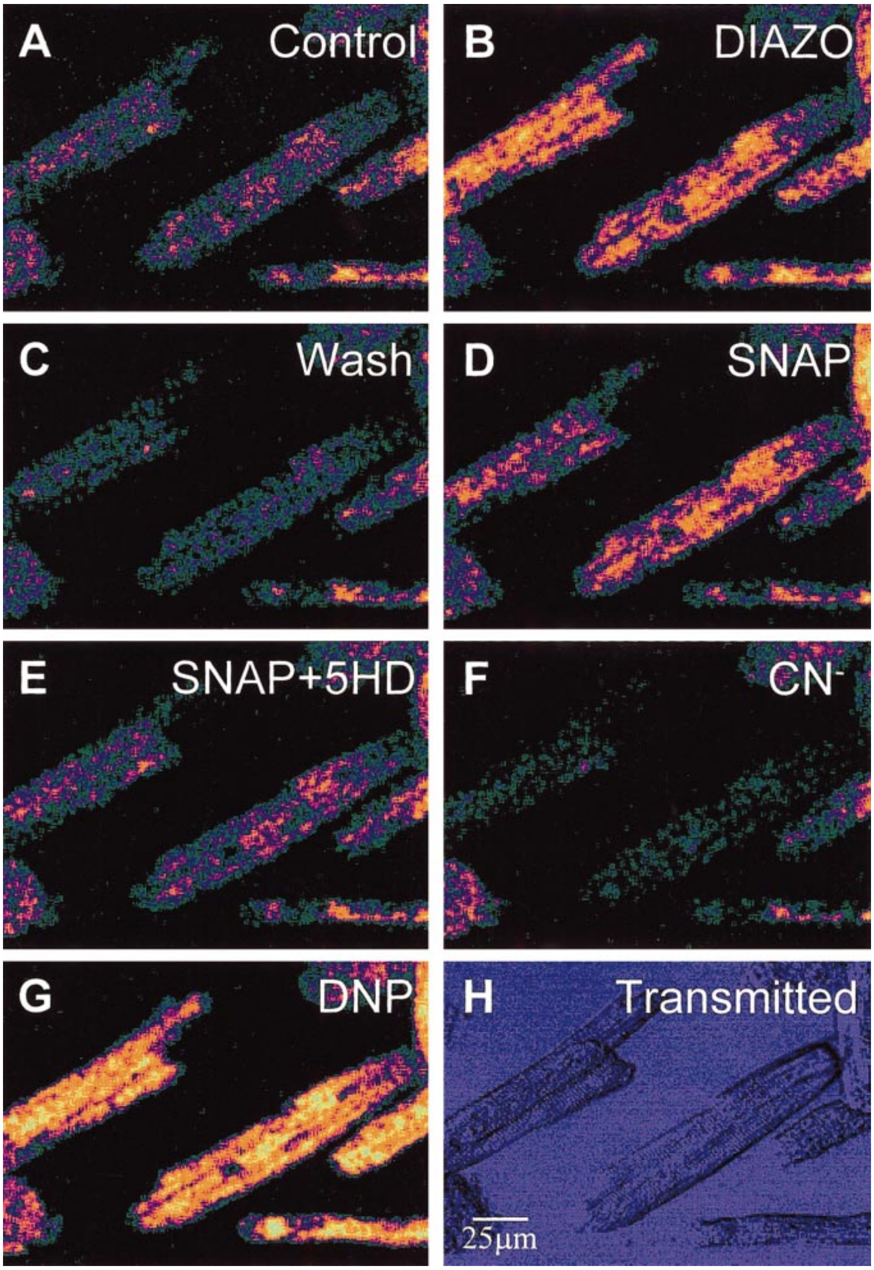

Figure 2. Confocal imaging confirms mitochondria-oxidizing effect of SNAP. A pseudocolor palette was applied to visualize relative increase in mitochondrial flavoprotein, to yield images of cells at baseline (Control, A), after 7 minutes' exposure to diazoxide (100 μmol/L, B), washing out diazoxide (Wash, C), after 12 minutes' exposure to SNAP (200 μmol/L, D), and additional application of 1 mmol/L 5HD (SNAP+5HD, E). Dynamic range of flavoprotein fluorescence is indicated by exposures to cyanide (4 mmol/L, F) and DNP (100 μmol/L, G) as minimum and maximum, respectively. H, transmitted light image of the same field.

those that would be expected from such an effect (in which case the mitochondrial matrix would have been reduced, as it is with CN^-).[18] Note also that the second exposure to diazoxide after SNAP increased flavoprotein oxidation above the levels reached in the first application, with less of a lag during the second exposure (4 minutes, versus 8 to 9 minutes during the first exposure).

To determine whether mitoK$_{ATP}$ channels are involved in SNAP-induced mitochondrial oxidation, we applied 5HD, a selective mitoK$_{ATP}$ channel blocker. Figure 1, B and C, shows that 1 mmol/L 5HD reversed (B) or prevented (C) the SNAP-induced flavoprotein oxidation. Figure 1D summarizes the amplitude of diazoxide-induced flavoprotein oxidation during the first [DIAZO(1)] and second exposures to diazoxide after the application of SNAP [DIAZO(2) SNAP]. Pretreatment with SNAP significantly enhanced the effects of diazoxide-induced oxidation; we have previously shown that repeated exposures to diazoxide alone do not produce potentiation.[17] Figure 1E summarizes the latency to mitoK$_{ATP}$ channel activation, measured as the time required to increase flavoprotein fluorescence to 20% of its maximal value after washing in diazoxide. The latency was significantly abbreviated during the second exposure to diazoxide after SNAP. Figure 1F summarizes the effects of 5HD on the SNAP-induced fluorescence changes and verifies that 5HD significantly and consistently inhibits SNAP-induced mitochondrial oxidation. These results indicate that SNAP-induced mitochondrial oxidation is mediated by activation of mitoK$_{ATP}$ channels.

Effects of SNAP on Flavoprotein Fluorescence Detected by Confocal Imaging

To further confirm the NO-induced activation of mitoK$_{ATP}$ channels, the effect of SNAP on flavoprotein fluorescence

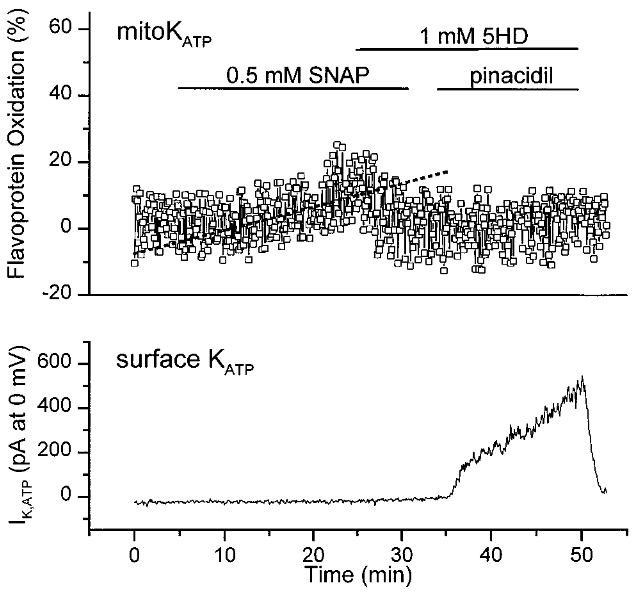

Figure 3. Effects of SNAP on flavoprotein fluorescence and $I_{K,ATP}$. Simultaneous measurement of flavoprotein fluorescence and $I_{K,ATP}$. Top, 0.5 mmol/L SNAP induced flavoprotein oxidation without preexposure to diazoxide. Bars indicate periods when cells were exposed to each drug. Note that 1 mmol/L 5HD inhibited oxidative effects of SNAP and 100 μmol/L pinacidil. Bottom, Time course of whole-cell current measured at 0 mV; pinacidil activated $I_{K,ATP}$ even in presence of 5HD.

was measured by confocal imaging. Fluorescence was low under control conditions (Figure 2A), but exposure to diazoxide reversibly increased fluorescence (B; washout image in C). Subsequent exposure to SNAP also increased flavoprotein fluorescence (D), but SNAP-induced oxidation was inhibited by additional application of 5HD (E). Images were calibrated at the end of the experiment by exposure to cyanide (F) and DNP (G). The patchy distribution of fluorescence in the confocal images is typical of mitochondria,[17,18] confirming that NO oxidizes the mitochondrial matrix by activation of mitoK$_{ATP}$ channels.

Effects of SNAP on mitoK$_{ATP}$ and Sarcolemmal K$_{ATP}$ Channels

To test the selectivity of NO on mitoK$_{ATP}$ versus sarcolemmal K$_{ATP}$ channels, we examined the effects of SNAP on flavoprotein fluorescence and whole-cell currents simultaneously. In Figure 3, application of 0.5 mmol/L SNAP without preexposure to diazoxide gradually oxidized the mitochondrial matrix with a slope of 0.78%/min. SNAP-induced oxidation was inhibited by coapplication of 1 mmol/L 5HD. In the continued presence of 5HD, subsequent exposure to 100 μmol/L pinacidil (a mixed mitoK$_{ATP}$/surface K$_{ATP}$ agonist)[16] failed to induce mitochondrial oxidation. In contrast, Figure 3 (bottom) shows that SNAP had no effect on sarcolemmal K$_{ATP}$ channels, because pinacidil activated sarcolemmal K$_{ATP}$ channels despite the presence of 5HD. These results are representative and reproducible. A 20-minute exposure to SNAP (0.5 mmol/L) had no significant effect on whole-cell current (before, 5.6±6.1 pA versus after, 12.9±4.8 pA at 0 mV, n=4, P=NS). Nevertheless, in the presence of 1 mmol/L 5HD, a 10-minute exposure to 100 μmol/L pinacidil increased sarcolemmal K$_{ATP}$ current (554.9±82.9 pA at 0 mV, n=4, P<0.001 versus before). These results indicate that SNAP selectively activates mitoK$_{ATP}$ channels. Furthermore, Figure 3 demonstrates that SNAP-induced activation of mitoK$_{ATP}$ channels does not require preexposure to diazoxide. Finally, the finding that 5HD suppresses the mitochondrial oxidation induced by pinacidil, but not the agonist effect on $I_{K,ATP}$,

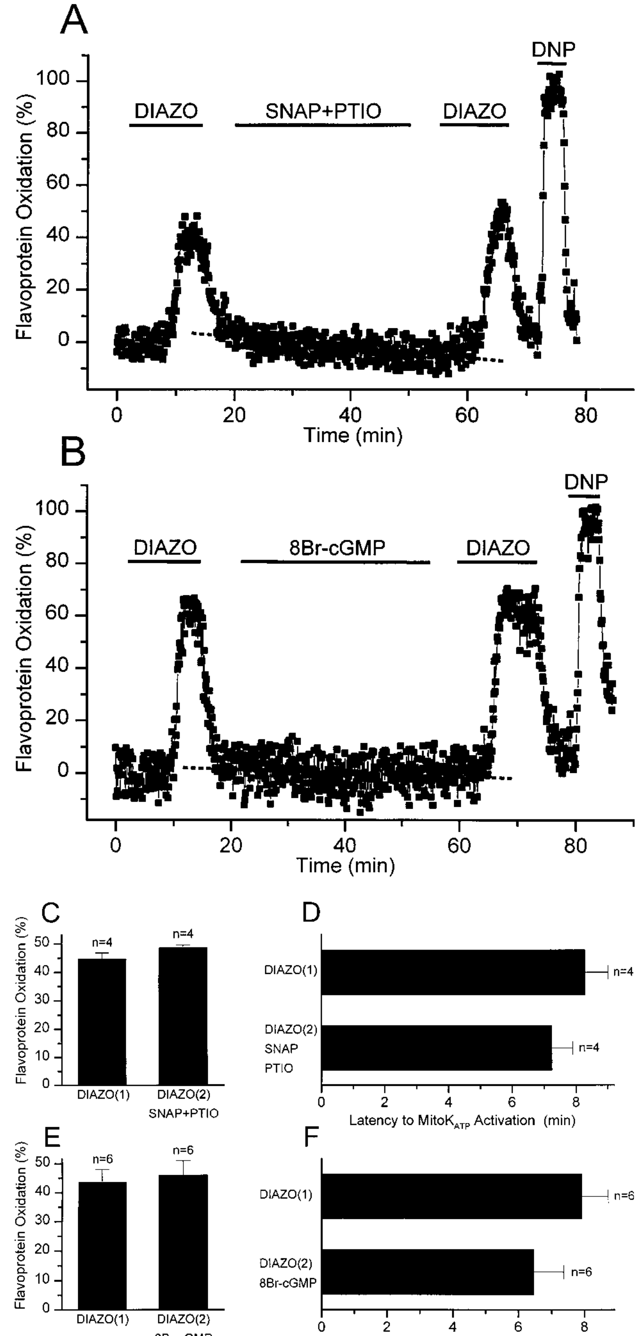

Figure 4. Changes in flavoprotein oxidation induced by coapplication of carboxy-PTIO (100 μmol/L) with SNAP (100 μmol/L) (A) and 1 mmol/L 8Br-cGMP (B) in a cell twice exposed to diazoxide. C and D summarize data for percentage of diazoxide-induced flavoprotein oxidation and latency of mitoK$_{ATP}$ channel activation measured in first [DIAZO(1)] and second exposure after coapplication of carboxy-PTIO with SNAP [DIAZO(2) SNAP+PTIO]. E and F summarize analogous data for 8Br-cGMP.

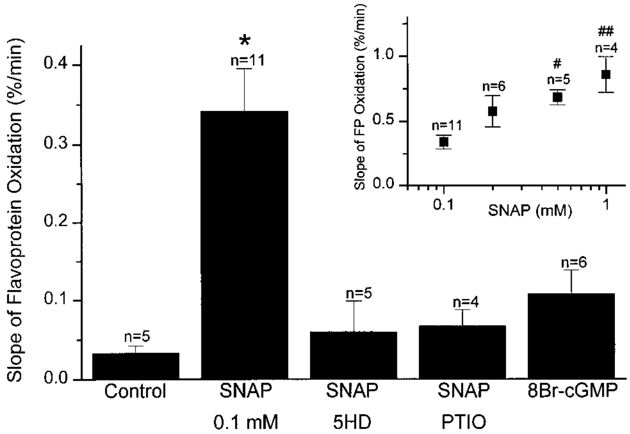

Figure 5. Summarized data for slope of flavoprotein oxidation during exposure to various pharmacological agents as indicated. *$P<0.01$ vs Control. Inset, Dose-response relationship for SNAP (0.1, 0.2, 0.5, and 1 mmol/L). #$P<0.05$, ##$P<0.01$ vs 0.1 mmol/L SNAP.

demonstrates that 1 mmol/L 5HD is a selective inhibitor of mitoK$_{ATP}$ channels in rabbit ventricular cells.[16,17]

Mediation by NO Independent of cGMP

To verify that the SNAP-induced changes are actually mediated by the release of NO, we tested the effects of carboxy-PTIO, an NO scavenger,[21] on the SNAP-induced flavoprotein oxidation. Figure 4A shows that coapplication of carboxy-PTIO with SNAP prevented the flavoprotein oxidation (slope <0%/min). Because many (but not all) of the effects of NO occur via a cGMP-dependent pathway,[22,23] we tested whether NO-induced activation of mitoK$_{ATP}$ is mimicked by 8Br-cGMP. Figure 4B shows that exposure to this cell-permeable cGMP analogue did not increase flavoprotein oxidation, nor did pretreatment with 8Br-cGMP enhance diazoxide-induced oxidation. The effects of the NO scavenger and 8Br-cGMP were observed reproducibly. Figure 4, C and D, shows that carboxy-PTIO abolished the enhancing effects of SNAP on diazoxide-induced oxidation, confirming that the SNAP-induced change is mediated by release of NO. Figure 4, E and F, summarizes data for 8Br-cGMP, confirming that it fails to mimic the effects of SNAP.

The pooled data in Figure 5 reveal that SNAP significantly increases the slope of percent change in flavoprotein oxidation and that the SNAP-induced effect is inhibited by 5HD and carboxy-PTIO. The inset shows the dose-response relationship between SNAP concentration and flavoprotein oxidation. Taken together with the results in Figure 4, these experiments support the idea that SNAP activates mitoK$_{ATP}$ channels dose-dependently via a direct effect of NO, not mediated by cGMP.

Effects of SNAP in the Presence of Diazoxide

We previously reported that protein kinase C (PKC) activation enhances diazoxide-induced changes without affecting basal flavoprotein fluorescence.[16] This finding indicates that the modulation of mitoK$_{ATP}$ by PKC may depend on whether the channels are in the open or closed state when the kinase becomes active. To test for analogous state-dependent changes in the case of NO, we quantified the effects of SNAP on channels that had already been opened by diazoxide. Figure 6A shows that 1 mmol/L SNAP rapidly enhanced diazoxide-induced oxidation when applied after the effect of diazoxide had reached steady state. Note that in this case, the effects of SNAP were reversible. Figure 6C shows that carboxy-PTIO abolished the enhancing effect of SNAP on diazoxide-induced oxidation, and Figure 6E demonstrates that 8Br-cGMP failed to mimic the effects of SNAP on mitoK$_{ATP}$ in the presence of diazoxide. Figure 6, B, D, and F, summarizes data for coadministration of diazoxide with SNAP, SNAP+carboxy-PTIO, and 8Br-cGMP, respectively. These results indicate that NO enhances mitoK$_{ATP}$ channels preactivated by diazoxide. Channels that are already open appear to be more susceptible to the potentiating actions of NO than channels that are in the closed state.

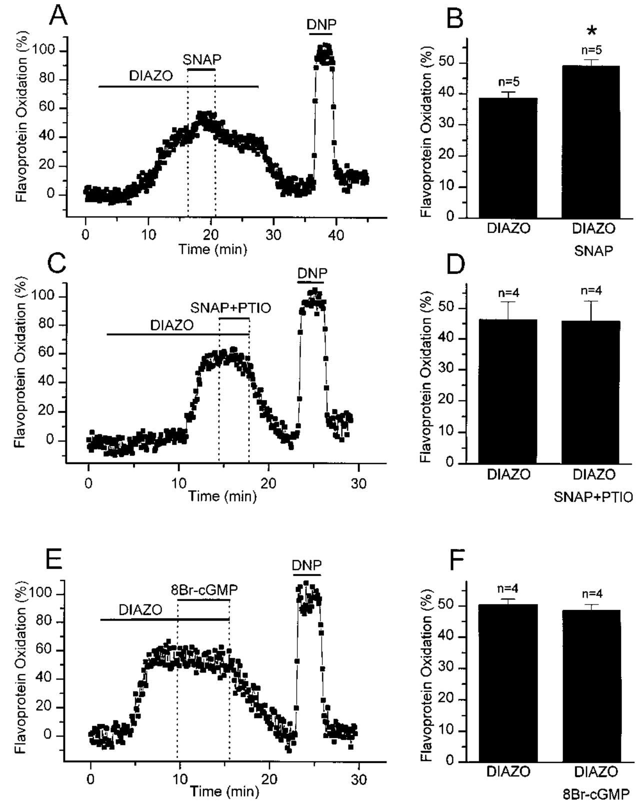

Figure 6. Coapplication of diazoxide and SNAP. A, 100 μmol/L diazoxide-induced flavoprotein oxidation in additional application of 1 mmol/L SNAP. B summarizes data for diazoxide-induced oxidation in absence (left) and presence (right) of SNAP (1 mmol/L). C and E show time course of diazoxide-induced change in additional application of SNAP (1 mmol/L) with carboxy-PTIO (100 μmol/L) and 8Br-cGMP (1 mmol/L), respectively. D and F summarize data for effects of coapplication of diazoxide with each of several pharmacological agents as indicated. *$P<0.05$ vs DIAZO.

Discussion

Our data reveal that NO selectively activates mitoK$_{ATP}$ channels but not sarcolemmal K$_{ATP}$ channels. The modulation of mitoK$_{ATP}$ channels by NO is manifested in 2 ways. One is the gradual oxidation induced by NO alone, and the other is potentiation of mitoK$_{ATP}$ channels preopened by diazoxide.

The latter effect resembles that of phorbol esters that turn on PKC and enhance diazoxide-induced oxidation,[16] but unlike NO, phorbol esters alone do not suffice to activate the channels. It is possible that the 2 observations share a common pathway, inasmuch as reactive oxygen species (such as the NO product peroxynitrite) are known to activate PKC.[13,24,25] Shinbo and Iijima[26] reported that the application of the NO donor NOR-3 increased the open probability of agonist-activated surface K_{ATP} channels in cardiac myocytes, whereas NOR-3 had no effect in the absence of channel agonists. We have found that PKC primes ventricular surface K_{ATP} channels to open in response to agonists or to metabolic inhibition, but basal activity is unaffected.[27,28] These findings again resemble the rapid enhancement of mitoK_{ATP} by NO in the presence of diazoxide but differ in the inability of the modulator to alter basal activity. Although the structural relationship between surface and mitoK_{ATP} channels is unknown,[29] the results suggest that the 2 channels may share regulatory pathways sensitive to NO, as is the case for PKC.[16,27,29] In pancreatic β cells, NO has been argued to inhibit glycolysis, leading to a secondary activation of surface K_{ATP} channels.[30] However, SNAP did not increase whole-cell K_{ATP} current in the present study, consistent with the finding that NO alone did not activate single K_{ATP} channels in cell-attached mode in guinea pig ventricular myocytes.[26] The mitoK_{ATP} channel is not especially sensitive to changes in bulk [ATP], remaining closed even at 0.5 mmol/L [ATP].[31] Considering these observations, it seems unlikely that the effects of NO on mitoK_{ATP} channels reflect inhibition of glycolysis.

Both NO and mitoK_{ATP} channels have been implicated in the delayed phase of preconditioning known as the "second window" of protection.[11–13] MitoK_{ATP} channel opening is cardioprotective during ischemia,[17,32] whereas blockade of mitoK_{ATP} channels abolishes both classic and second-window protection. The present study establishes NO as an endogenous mitoK_{ATP} channel opener that may be able to recruit cardioprotection in the second window. NO may play a particularly prominent role in the second window because of changes in gene expression, notably the upregulation of nitric oxide synthase that occurs within 24 hours of conditioning ischemia. Although the relationships between mitoK_{ATP} channel activation and cardioprotection remain elusive, the opening of channels in the inner membrane may dissipate the mitochondrial potential established by the proton pump, perhaps blunting the Ca^{2+} overload that would otherwise occur as a result of the large driving force for Ca^{2+} entry into mitochondria during ischemia.[16,17] It was recently reported that mitoK_{ATP} channel openers release Ca^{2+} from Ca^{2+}-loaded mitochondria.[33] The uncoupling by diazoxide appears to be much gentler than that which can be induced by agents such as DNP[34]; indeed, severe uncoupling should be harmful to myocytes, because energy production is critically reduced. We speculate that NO, functioning as an endogenous mitoK_{ATP} channel opener, may titrate the coupling level of the mitochondria to an optimum that blunts mitochondrial calcium overload without significantly undermining ATP synthetic capacity.

Acknowledgments

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References

1. Zweier JL, Wang P, Kuppusamy P. Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. *J Biol Chem*. 1995;270:304–307.
2. Giulivi C, Poderoso JJ, Boveris A. Production of nitric oxide by mitochondria. *J Biol Chem*. 1998;273:11038–11043.
3. Giulivi C. Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. *Biochem J*. 1998;332:673–679.
4. Nakanishi K, Vinten-Johansen J, Lefer DJ, Zhao Z, Fowler WC III, McGee S, Johnston WE. Intracoronary L-arginine during reperfusion improves endothelial function and reduces infarct size. *Am J Physiol*. 1992;263:H1650–H1658.
5. Williams MW, Taft CS, Ramnauth S, Zhao ZQ, Vinten-Johansen J. Endogenous nitric oxide protects against ischemia-reperfusion injury in the rabbit. *Cardiovasc Res*. 1995;30:79–86.
6. Hartman JC, Kurc GM, Hullinger TG, Wall TM, Sheehy RM, Shebuski RJ. Inhibition of nitric oxide synthase prevents myocardial protection by ramiprilat. *J Pharmacol Exp Ther*. 1994;270:1071–1076.
7. Vegh A, Szekeres L, Parratt J. Preconditioning of the ischaemic myocardium: involvement of the L-arginine nitric oxide pathway. *Br J Pharmacol*. 1992;107:648–652.
8. Tosaki A, Maulik N, Elliott GT, Blasig IE, Engelman RM, Das DK. Preconditioning of rat heart with monophosphoryl lipid A: a role for nitric oxide. *J Pharmacol Exp Ther*. 1998;285:1274–1279.
9. Woolfson RG, Patel VC, Neild GH, Yellon DM. Inhibition of nitric oxide synthesis reduces infarct size by an adenosine-dependent mechanism. *Circulation*. 1995;91:1545–1551.
10. Weselcouch EO, Baird AJ, Sleph P, Grover GJ. Inhibition of nitric oxide synthesis does not affect ischemic preconditioning in isolated perfused rat hearts. *Am J Physiol*. 1995;268:H242–H249.
11. Takano H, Tang XL, Qiu Y, Guo Y, French BA, Bolli R. Nitric oxide donors induce late preconditioning against myocardial stunning and infarction in conscious rabbits via an antioxidant-sensitive mechanism. *Circ Res*. 1998;83:73–84.
12. Bolli R, Manchikalapudi S, Tang XL, Takano H, Qiu Y, Guo Y, Zhang Q, Jadoon AK. The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase: evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. *Circ Res*. 1997;81:1094–1107.
13. Bolli R, Dawn B, Tang XL, Qiu Y, Ping P, Xuan XT, Jones WK, Takano H, Guo Y, Zhang J. The nitric oxide hypothesis of late preconditioning. *Basic Res Cardiol*. 1998;93:325–328.
14. Benjamin IJ, McMillan DR. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res*. 1998;83:117–132.
15. Bernardo NL, D'Angelo M, Okubo S, Joy A, Kukreja RC. Delayed ischemic preconditioning is mediated by opening of ATP-sensitive potassium channels in the rabbit heart. *Am J Physiol*. 1999;276:H1323–H1330.
16. Sato T, O'Rourke B, Marbán E. Modulation of mitochondrial ATP-dependent K^+ channels by protein kinase C. *Circ Res*. 1998;83:110–114.
17. Liu Y, Sato T, O'Rourke B, Marbán E. Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation*. 1998;97:2463–2469.
18. Romashko DN, Marbán E, O'Rourke B. Subcellular metabolic transients and mitochondrial redox waves in heart cells. *Proc Natl Acad Sci U S A*. 1998;95:1618–1623.
19. Poderoso JJ, Carreras MC, Lisdero C, Riobo N, Schopfer F, Boveris A. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch Biochem Biophys*. 1996;328:85–92.
20. Cleeter MWJ, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AHV. Reversible inhibition of cytochrome c oxidase, the terminal

enzyme of the mitochondrial respiratory chain, by nitric oxide. *FEBS Lett*. 1994;345:50–54.
21. Akaike T, Yoshida M, Miyamoto Y, Sato K, Kohno M, Sasamoto K, Miyazaki K, Ueda S, Maeda H. Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/ · NO through a radical reaction. *Biochemistry*. 1993;32:827–832.
22. Mery PF, Lohmann SM, Walter U, Fischmeister R. Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc Natl Acad Sci U S A*. 1991;88:1197–1201.
23. Wahler GM, Dollinger SJ. Nitric oxide donor SIN-1 inhibits mammalian cardiac calcium current through cGMP-dependent protein kinase. *Am J Physiol*. 1995;37:C45–C54.
24. Gopalakrishna R, Anderson WB. Ca^{2+}- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain. *Proc Natl Acad Sci U S A*. 1989;86: 6758–6762.
25. Brawn MK, Chiou WJ, Leach KL. Oxidant-induced activation of protein kinase C in UC11 MG cells. *Free Radic Res*. 1995;22:23–37.
26. Shinbo A, Iijima T. Potentiation by nitric oxide of the ATP-sensitive K^+ current induced by K^+ channels openers in guinea-pig ventricular cells. *Br J Pharmacol*. 1997;120:1568–1574.
27. Liu Y, Gao WD, O'Rourke B, Marbán E. Priming effect of adenosine on K_{ATP} currents in intact ventricular myocytes: implications for preconditioning. *Am J Physiol*. 1997;273:H1637–H1643.
28. Liu Y, Gao WD, O'Rourke B, Marbán E. Synergic modulation of ATP-sensitive K^+ currents by protein kinase C and adenosine: implications for ischemic preconditioning. *Circ Res*. 1996;78:443–454.
29. Hu H, Sato T, Seharaseyon J, Liu Y, Johns DC, O'Rourke B, Marbán E. Pharmacological and histochemical distinctions between molecularly-defined sarcolemmal K_{ATP} channels and native cardiac mitochondrial K_{ATP} channels. *Mol Pharmacol*. 1999;55:1000–1005.
30. Tsuura Y, Ishida H, Hayashi S, Sakamoto K, Horie M, Seino Y. Nitric oxide opens ATP-sensitive K^+ channels through suppression of phosphofructokinase activity and inhibits glucose-induced insulin release in pancreatic β cells. *J Gen Physiol*. 1994;104:1079–1099.
31. Garlid KD, Paucek P, Yarov-Yarovoy V, Sun X, Schindler PA. The mitochondrial K_{ATP} channel as a receptor for potassium channel openers. *J Biol Chem*. 1996;271:8796–8799.
32. Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA, Grover GJ. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels: possible mechanism of cardioprotection. *Circ Res*. 1997;81:1072–1082.
33. Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, Terzic A. Mitochondrial ATP-sensitive K^+ channels modulate cardiac mitochondrial function. *Am J Physiol*. 1998;275:H1567–H1576.
34. Grimmsmann T, Rustenbeck I. Direct effect of diazoxide on mitochondria in pancreatic β-cells and on isolated liver mitochondria. *Br J Pharmacol*. 1998;123:781–788.