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Abstract— cADP-ribose (cADPR) induces the release of Ca^{2+} from the intracellular stores of coronary artery smooth muscle cells. However, little is known about the role of cADPR-mediated intracellular Ca^{2+} release in the control of vascular tone. The present study examined the effects of nicotinamide, a specific inhibitor of ADP-ribosylcyclase, on the vascular tone of bovine coronary arteries. A bovine coronary artery homogenate stimulated the conversion of nicotinamide guanine dinucleotide into cGDP-ribose, which is a measure of ADP-ribosylcyclase activity. Nicotinamide significantly inhibited the formation of cGDP-ribose in a concentration-dependent manner: at a concentration of 10 mmol/L, it reduced the conversion rate from 3.34 ± 0.11 nmol $\cdot$ $min^{-1} \cdot mg^{-1}$ of protein in control cells to 1.42 ± 0.11 nmol $\cdot$ $min^{-1} \cdot mg^{-1}$ of protein in treated cells, a 58% reduction. In U46619-precontracted coronary artery rings, nicotinamide produced concentration-dependent relaxation. Complete relaxation with nicotinamide occurred at a dose of 8 mmol/L; the median inhibitory concentration (IC_{50}) was 1.7 mmol/L. In the presence of a cell membrane–permeant cADPR antagonist, 8-bromo-cADPR, nicotinamide-induced vasorelaxation was markedly attenuated. Pretreatment of the arterial rings with ryanodine (50 μmol/L) significantly blunted the vasorelaxation response to nicotinamide. However, iloprost- and adenosine-induced vasorelaxation was not altered by 8-bromo-cADPR. Moreover, nicotinamide significantly attenuated KCl- or Bay K8644–induced vasoconstriction by 60% and 70%, respectively. These results suggest that the inhibition of cADPR formation by nicotinamide produces vasorelaxation and blunts KCl- and Bay K8644–induced vasoconstriction in coronary arteries and that the cADPR-mediated Ca^{2+} signaling pathway plays a role in the control of vascular tone in coronary circulation. **(*Hypertension*. 2000;35[part 2]:397-402.)**

Key Words: adenosine diphosphate ribose ■ arteries ■ calcium channels ■ niacinamide

cADP-ribose (cADPR), an endogenous metabolite of nicotinamide adenine dinucleotide, induces the mobilization of Ca^{2+} from ryanodine-sensitive stores in a wide variety of tissues.[1–6] The mechanism by which cADPR causes the mobilization of this intracellular calcium is completely independent of the action of inositol triphosphate. This cADPR-mediated Ca^{2+} signaling participates in the regulation of a variety of cell functions, including insulin secretion, egg fertilization, cell proliferation, cardiac excitation-contraction coupling, and the effects of nitric oxide (NO) in nonmuscle tissue.[1–6] Recent studies indicated that cADPR induces Ca^{2+} release from the intracellular stores of coronary artery smooth muscle cells.[7] However, little is known of the role of cADPR-mediated Ca^{2+} mobilization in the control of vascular tone.

Nicotinamide, an amide derivative of vitamin B_3, is a potent vasodilator.[8,9] Numerous studies have demonstrated that nicotinamide increases the perfusion and oxygenation of tumors, thereby increasing the sensitivity of the tumors to radio- or chemical therapies.[10–13] However, the mechanism by which nicotinamide produces vasodilation and increases tissue perfusion is poorly understood. Recently, nicotinamide was reported to inhibit the activity of purified ADP-ribosylcyclase and to decrease cADPR production.[14,15] In sea urchin eggs, nicotinamide blocked the Ca^{2+}-mobilizing action of β-nicotinamide adenine dinucleotide (+), cGMP, and NO by inhibiting ADP-ribosylcyclase activity.[16]

We wondered whether nicotinamide alters cADPR production in coronary artery smooth muscle cells and whether nicotinamide-induced vasodilation is associated with the inhibition of ADP-ribosylcyclase activity. The present study was designed to answer these questions. It tested the hypothesis that cADP-ribose-mediated Ca^{2+} signaling plays a role in the control of vascular tone in coronary circulation and that the inhibition of cADPR-mediated Ca^{2+} signaling produces vasorelaxation. First, we determined the effects of nicotinamide on the enzyme activity involved in the production of cADPR in the coronary arteries. Then, we examined the vasorelaxation response of the coronary arteries to nicotinamide in the absence or presence of a cADPR antagonist, 8-bromo-cADPR (8-Br-cADPR). Because cADPR-mediated intracellular Ca^{2+} mobilization is associated with the activation of ryanodine receptors, we also determined the effect of blocking the ryanodine receptors in nicotinamide-mediated

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vasorelaxation. To address the role of endogenous cADPR in mediating vasoconstriction, we examined the effect of nicotinamide on the vasoconstriction induced by KCl depolarization and the Ca^{2+} channel activator, Bay K8644.

Methods

Preparation of Homogenate from Small Bovine Coronary Arteries

Coronary artery homogenates were prepared as described previously.[17,18] Briefly, bovine hearts were obtained from a local slaughterhouse. Small coronary arteries were microdissected under a dissecting stereomicroscope. These arteries were pooled and stored in ice-cold PBS. The dissected coronary arteries were cut into very small pieces and homogenized with a glass homogenator in ice-cold HEPES buffer containing (in mmol/L): Na-HEPES 25, EDTA 1, sucrose 255, and phenylmethylsulfonyl fluoride 0.1. After centrifuging the homogenized tissue at 6000*g* for 5 minutes at 4°C, the supernatant containing the membrane and cytosolic components (which was termed the homogenate) was separated into aliquots, frozen in liquid N_2, and stored at −80°C until used.

Assay of ADP-Ribosylcyclase in Bovine Coronary Artery Homogenates

To determine the activity of ADP-ribosylcyclase, the homogenates (50 μg) were incubated for 60 minutes with 1 mmol/L nicotinamide guanine dinucleotide (NGD) at 37°C in an assay buffer containing (in mmol/L): potassium gluconate 250, N-methylglucamine 250, HEPES 20, and $MgCl_2$ 1 (pH 7.2). NGD was used as a substrate to determine ADP-ribosylcyclase activity because ADP-ribosylcyclase converts NGD into cGDP-ribose (cGDPR). Unlike cADPR, cGDPR cannot be hydrolyzed by cADPR hydrolase. A conversion rate of NGD into cGDPR more precisely represents ADP-ribosylcyclase activity. The total reaction volume was 0.1 mL. The reaction mixture was then rapidly frozen in liquid N_2 to terminate the reaction. Before analysis with high-performance liquid chromatography (HPLC), the reaction mixtures were centrifuged at 4°C using an Amicon microultrafilter at 13 000 rpm for 10 minutes to remove the proteins.

The reaction products in the ultrafiltrate were analyzed by a HPLC system using a Hewlett-Packard 1090L solvent delivery system and a 1046A programmable fluorescence detector (Hewlett Packard). The excitation wavelength was 300 nm, and the emission wavelength was 410 nm. Nucleotides were resolved on a Supelcosil LC-18 (3 μm; 4.6×150 mm) column (Supelco). The injection volume was 20 μL. The mobile phase consisted of 150 mmol/L ammonium acetate (pH 5.5), which contained either 5% methanol (solvent A) or 50% methanol (solvent B). The nucleotides were separated in solvent A with a gradient of 0% solvent B to 25% solvent B for 12 minutes, 25% solvent B to 100% solvent B for 4 minutes, and then 0% solvent B for 5 minutes after 4 minutes. The flow rate was 0.8 mL/min.[17] Peak identities were confirmed by comigration with known standards. Quantitative measurements were performed by comparing known concentrations of standards.

Vascular Reactivity Studies

Vascular reactivity in bovine coronary arteries was determined as previously described by our laboratory.[19] Briefly, the epicardial left anterior descending coronary artery was dissected, cleaned of adhering fat and connective tissue, and placed in Krebs bicarbonate solution, which contained (in mmol/L): NaCl 119, KCl 5, $NaHCO_3$ 24, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11, EDTA 0.02, and $CaCl_2$ 3.2. The rings were prepared and suspended in a 6-mL water-jacked organ chamber at 37°C. The contractile responses were monitored using a computerized polygraphic recording system. After an equilibration period of 1.5 hours, the vessels were activated by adding KCl (80 mmol/L) until reproducible contractions were obtained. Then, 1 ring of each pair received vehicle (0.01% ethanol) and the other ring received 8-Br-cADPR (30 μmol/L; n=12), tetracaine (20 μmol/L; n=12), or ryanodine (50 μmol/L; n=12) for 10 minutes before the addition of the thromboxane mimetic, U46619 (20 nmol/L). 8-Br-cADPR selectively blocks cADPR-induced Ca^{2+} release in different cells. This cADPR antagonist is cell-membrane permeant.[3] At high concentrations (>10 μmol/L), ryanodine blocks ryanodine receptors and, thereby, abolishes Ca^{2+} release from the ryanodine-sensitive pool.[7] U46619 was selected as the precontracting agent because it produced reproducible, sustained contractions in coronary arteries.[19] After a sustained contraction by U46619 was obtained, cumulative additions of nicotinamide (0.5 to 10 mmol/L), iloprost (10^{-8} to 10^{-4} mol/L), or adenosine (10^{-7} to 10^{-3} mol/L) were made every 4 minutes until a plateau response was reached. Results were expressed as the percent relaxation relative to the U46619 contraction, with 100% relaxation reaching the basal tension before U46619 contraction.

The effects of nicotinamide on the vasoconstriction induced by KCl and the Ca^{2+}-channel agonist Bay K8644 were examined using the same arterial preparation but without U46619 precontraction. KCl (20 to 80 mmol/L) was added to the bath by replacing the solution containing low NaCl, and Bay K8644 was directly added into the bath. The percent of accumulative contraction effects was recorded and calculated with 100% contraction by 80 mmol/L KCl.

Statistical Analysis

Data are presented as mean±SEM. The significance of differences in mean values within and between multiple groups was examined using ANOVA for repeated measures followed by a Duncan's multiple range test. Student's *t* test was used to examine the significance of difference in 2 groups. $P<0.05$ was considered significant.

Results

Effect of Nicotinamide on the Activity of ADP-Ribosylcyclase

Figure 1 presents a representative reverse-phase HPLC chromatogram depicting the production of cGDPR by ADP-ribosylcyclase in coronary artery homogenates and the effect of the ADP-ribosylcyclase inhibitor nicotinamide on the production of cGDPR. When the homogenates were incubated with NGD, a product with a retention time of 2.1 coeluted with synthetic cGDPR (Figure 1A). In the presence of nicotinamide (8 mmol/L), cGDPR production was markedly decreased (Figure 1B). The conversion rate of NGD into cGDPR averaged 3.34 nmol · min^{-1} · mg^{-1} of protein. Nicotinamide produced a concentration-dependent decrease in the activity of ADP-ribosylcyclase. The conversion rate of NGD into cGDPR was decreased to 1.42 nmol · min^{-1} · mg^{-1} of protein, which represents a 58% reduction, when 10 mmol/L nicotinamide was added to the reaction mixture (Figure 1C).

Nicotinamide-Induced Relaxation of Bovine Coronary Arteries

Figure 2A presents a typical recording of coronary artery reactivity to nicotinamide in the absence and presence of the cADPR antagonist 8-Br-cADPR. Nicotinamide produced a concentration-dependent relaxation in U46619-precontracted coronary artery rings, and 8-Br-cADPR attenuated the vasorelaxation to nicotinamide. Figure 2B summarizes the relaxation responses to nicotinamide. The maximal relaxation to nicotinamide occurred at 6 mmol/L; the median inhibitory concentration (IC_{50}) was 1.7 mmol/L. Pretreatment of coronary artery rings with the cADPR antagonist 8-Br-cADPR markedly attenuated

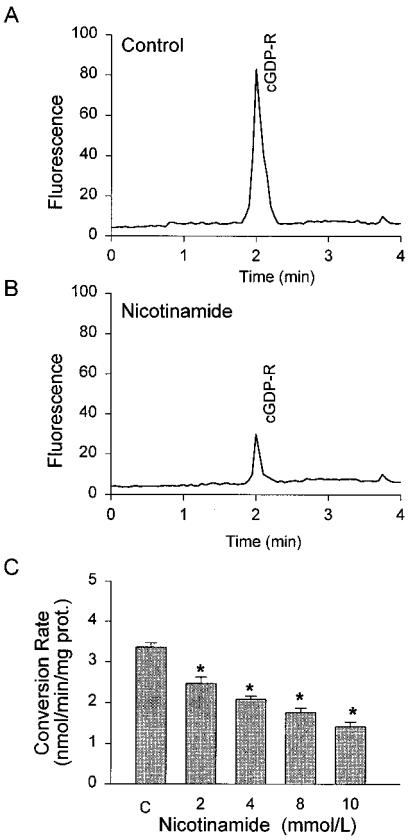

Figure 1. HPLC analysis of cGDPR produced by homogenates of bovine coronary arteries. A, Typical fluorescence HPLC chromatogram showing production of cGDPR (control). B, Production of cGDPR in coronary artery homogenates in presence of nicotinamide (10 mmol/L). C, Summarized data showing concentration-dependent inhibition of conversion rate of NGD into cGDPR, which represents activity of ADP-ribosylcyclase in coronary arteries. Values in C are mean±SE (n=8). *Significant difference from control ($P<0.05$). Prot indicates protein.

nicotinamide-induced relaxation. The concentration-relaxation curve to nicotinamide was shifted to the right. Only 52% relaxation was observed at a nicotinamide concentration of 8 mmol/L (Figure 2B).

Similar to the effect of 8-Br-cADPR, ryanodine also significantly blunted the vasorelaxation response of coronary arteries to nicotinamide. In the presence of ryanodine (Figure 3), the nicotinamide-induced vasorelaxation response was significantly attenuated to an extent similar to that with 8-Br-cADPR. Pretreatment of the arteries with a combination of the 2 compounds attenuated nicotinamide-induced vasorelaxation only to an extent similar to that of each compound alone (data not shown).

Effect of 8-Br-cADPR on Iloprost- or Adenosine-Induced Vasorelaxation

As shown in Figure 4, iloprost and adenosine also produced concentration-dependent vasorelaxation in U46619-precontracted coronary arteries. Pretreatment of arterial rings with 8-Br-cADPR had no effect on iloprost- and adenosine-induced vasorelaxation.

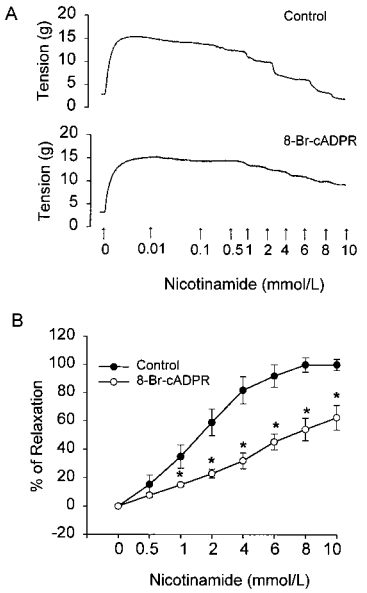

Figure 2. Vasorelaxation response of coronary arteries to nicotinamide. A, Typical recordings of coronary artery reactivity to nicotinamide in absence and presence of 8-Br-cADPR. B, Summary of data showing concentration-dependent relaxation induced by nicotinamide in absence and presence of 8-Br-cADPR in bovine coronary arteries. *Significant difference from values obtained during addition of nicotinamide alone (control) ($P<0.05$). Values are mean±SE (n=12).

Effect of Nicotinamide on KCl- and Bay K8644-Induced Vasoconstriction

As shown in Figure 5, KCl (20 to 80 mmol/L) and Bay K8644 (10 to 40 μmol/L) produced concentration-dependent vasoconstriction. At a concentration of 8 mmol/L, nicotinamide significantly attenuated the vasoconstrictor response to KCl and Bay K8644. Bay K8644–induced maximal vasoconstriction at 40 μmol/L was blocked by 70% in the presence of nicotinamide.

Discussion

The present study evaluated the effects of nicotinamide on cADPR production and vascular tone in small bovine coronary arteries. Nicotinamide inhibited ADP-ribosylcyclase activity in coronary artery homogenates, dilated U46619-precontracted coronary artery rings in a concentration-dependent manner, and blunted KCl- and Bay K8644–induced vasoconstriction. These results indicate that

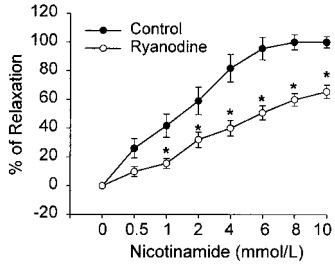

Figure 3. Effect of ryanodine on nicotinamide-induced relaxation of bovine coronary arteries in absence or presence of ryanodine. *Significant difference from values obtained during addition of nicotinamide alone (control) ($P<0.05$). Values are mean±SE (n=12).

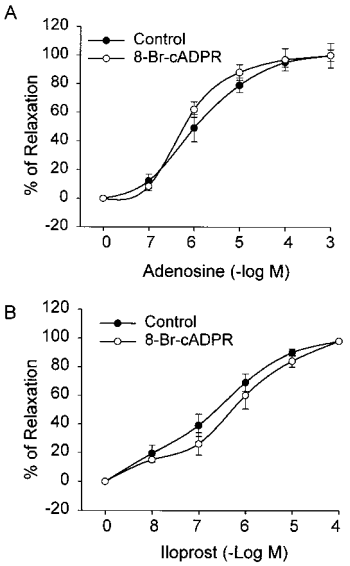

Figure 4. Effect of 8-Br-cADPR on iloprost- and adenosine-induced relaxation of bovine coronary arteries. A, Iloprost-induced vasorelaxation in absence or presence of 8-Br-cADPR. B, Adenosine-induced vasorelaxation in absence or presence of 8-Br-cADPR. Values are mean±SE (n=8).

endogenous cADPR may be involved in the control of coronary artery tone.

Recently, we reported that a metabolic pathway responsible for cADPR production and hydrolysis is present in coronary artery smooth muscle cells.[17,20] However, the physiological relevance of this pathway to the control of vascular tone remains unknown. In the present study, nicotinamide, which inhibits ADP-ribosylcyclase activity, was used to determine the effect of inhibiting endogenous cADPR production on coronary artery tone. First, HPLC analyses were performed to test the efficacy of nicotinamide in inhibiting cADPR production in coronary artery homogenates. The results showed that nicotinamide produced a concentration-dependent inhibition of ADP-ribosylcyclase activity, which is consistent with previous reports showing that nicotinamide inhibits the production of cADPR by purified ADP-ribosylcyclases[14,15] and sea urchin eggs.[16] These findings allowed us to use nicotinamide as a tool to test the role of endogenous cADPR in the control of vascular tone.

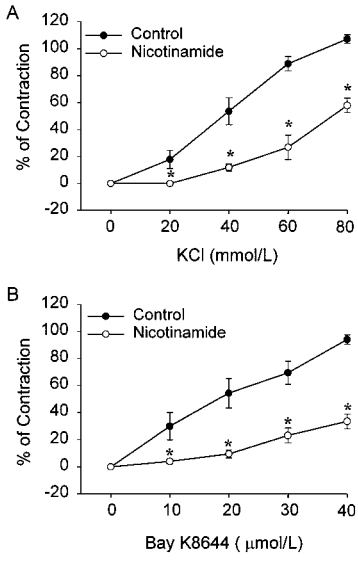

Figure 5. Effect of nicotinamide (6 mmol/L) on KCl- and Bay K8644–induced vasoconstriction. A, KCl-induced vasoconstriction in absence and presence of nicotinamide. B, Bay K8644-induced vasoconstriction in absence and presence of nicotinamide. *Significant difference from control ($P<0.05$). Values are mean±SE (n=8).

In U46619-precontracted coronary artery rings, nicotinamide produced concentration-dependent relaxation, with maximal relaxation to nicotinamide occurring at 6 mmol/L. These results support our hypothesis that the inhibition of endogenous cADPR leads to vasodilation in coronary arteries. The mechanism by which nicotinamide produces vasorelaxation has been studied, but it remains unknown. A recent study by Burns et al[9] examined the role of cAMP and cGMP in nicotinamide-induced vasorelaxation in rat tail arteries and demonstrated that selective inhibition of cAMP or cGMP production had no effect on nicotinamide-induced vasorelaxation. Increases in the levels of cAMP and cGMP by inhibiting phosphodiesterase also did not affect the vasorelaxation caused by nicotinamide. Burns et al[9] concluded that nicotinamide is unlikely to exert its main vasodilatory effect through enhancing the adenylate- or soluble guanylate cyclase–mediated pathways. Furthermore, nicotinamide reportedly inhibits mono-ADP-ribosyltransferase, a membrane-signaling enzyme; this may alter vascular tone through the ADP-ribosylation of various intracellular signaling or effector proteins in the coronary arteries. However, substantial evidence indicates that mono-ADP-ribosyltransferase is activated by NO, prostacyclin, or β-adrenergic agonists,[21–23] which are all potent vasodilators. These data suggest that the activation of mono-ADP-ribosyltransferase may result in vasodilation. More recently, we showed that endogenous mono-ADP-ribosyltransferase are present in the smooth muscle cells from bovine coronary arteries and that they mediate the effect of an endothelium-dependent hyperpolarizing factor, 11,12-epoxyeicosatrienoic acid, which activates Ca^{2+}-activated large-conductance potassium channels and produces vasodilation.[18] Therefore, the inhibition of ADP-ribosyltransferases by nicotinamide should result in vasoconstriction. It is unlikely that nicotinamide-induced vasorelaxation is caused by the inhibition of mono-ADP-ribosyltransferases.

To further define the role of cADPR in mediating nicotinamide-induced vasorelaxation, additional experiments were performed to examine the effects of the cADPR antagonist 8-Br-cADPR on the nicotinamide-induced vasorelaxation of coronary arteries. In these experiments, the arteries were pretreated with 8-Br-cADPR before contraction by U46619. In the presence of 8-Br-cADPR, any other inhibitors or blockers of cADPR should have a diminished effect if they share the same mechanism because the mechanism of action is already blocked. Indeed, we found that 8-Br-cADPR attenuated the vasodilator effect of nicotinamide, but not that of iloprost or adenosine, which affect the arteries through the production of cAMP.[19] This suggests that in the presence of 8-Br-cADPR, the action of cADPR is blocked so that this signaling pathway will not respond to stimuli that may

increase or decrease cADPR. It should be noted that this strategy was used in the present study because no cell-permeable agonist is available to restore the cADPR levels decreased by nicotinamide. However, this strategy is also widely used to study the mechanism of action of vasodilators. For instance, to determine the role of the renin-angiotensin system, ryanodine receptors, 20-HETE production, and Ca^{2+} channels in NO-induced vasodilation, the inhibitors of these systems have also been used. The vasodilator response to NO was attenuated by an angiotensin antagonist, ryanodine, an inhibitor of 20-HETE production, and Ca^{2+} blockers, suggesting that the action of NO is associated with these systems.[24–28]

Previous studies indicated that cADPR-induced Ca^{2+} release is associated with the activation of ryanodine receptors,[1–6] and ryanodine receptors exist in vascular smooth muscle cells.[29,30] We wondered whether ryanodine receptors are involved in nicotinamide-induced vasorelaxation. Ryanodine markedly attenuates the vasorelaxation response to nicotinamide. This suggests that nicotinamide may decrease cADPR levels and, consequently, reduce the activation of ryanodine receptors, thereby resulting in vasorelaxation.

The concentration of nicotinamide inhibiting ADP-ribosylcyclase seems greater than the concentration required for vasodilation. Specifically, a 50% reduction of ADP-ribosyl cyclase activity occurred at 8 mmol/L, but 50% vasorelaxation occurred at a concentration of 1.7 mmol/L nicotinamide. The reason for this inconsistency between the biochemical measurements and functional studies of vasodilation is unknown. These studies were performed under different conditions; therefore, they cannot be directly compared. The biochemical studies were performed in homogenates, whereas the tension studies were done in intact tissue and cells. Intracellular components in the homogenate may be different from those in the intact vessels, which may result in a low sensitivity to inhibition by nicotinamide. Another possibility is that the inhibition of the production of cADPR may not be the only mechanism that mediates nicotinamide-induced vasodilation. In fact, 8-Br-cADPR and ryanodine did not completely block nicotinamide-induced vasodilation. Comparing the control curves with those obtained using inhibitors (Figures 2B and 3), 50% vasorelaxation by nicotinamide (8 mmol/L) occurred in the presence of the inhibitors, suggesting that 50% of the relaxation is dependent on cADPR. This finding is consistent with 50% enzyme inhibition. The mechanism mediating cADPR-independent relaxation in coronary arteries remains to be determined.

In summary, the inhibition of the enzyme responsible for cADPR production by nicotinamide induced coronary artery vasorelaxation and blunted the vasoconstrictor response to membrane depolarization and Ca^{2+} channel activation. The blockade of the cADPR antagonist and ryanodine receptors attenuated nicotinamide-induced coronary vasorelaxation. These results indicate that the cADPR signaling pathway plays an important role in the control of coronary vascular tone and that the effects of cADPR are associated with the activation of ryanodine receptors.

Acknowledgments

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