Role of ADP-ribose in 11,12-EET-induced activation of $K_{\text{Ca}}$ channels in coronary arterial smooth muscle cells

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Am J Physiol Heart Circ Physiol 282: H1229–H1236, 2002. First published November 29, 2001; 10.1152/ajpheart.00736.2001.—We recently reported that cADP-ribose (cADPR) and ADP-ribose (ADPR) play an important role in the regulation of the Ca$^{2+}$-activated K$^+$ ($K_{\text{Ca}}$) channel activity in coronary arterial smooth muscle cells (CASMCs). The present study determined whether these novel signaling nucleotides participate in 11,12-epoxyeicosatrienoic acid (11,12-EET)-induced activation of the $K_{\text{Ca}}$ channels in CASMCs. HPLC analysis has shown that 11,12-EET increased the production of ADPR but not the formation of cADPR. The increase in ADPR production was due to activation of NAD glycohydrolase as measured by a conversion rate of NAD into ADPR. The maximal conversion rate of NAD into ADPR in coronary homogenate was increased from 2.5 ± 0.2 to 3.4 ± 0.3 nmol·min$^{-1}$·mg protein$^{-1}$ by 11,12-EET. The regioisomers of 8,9-EET, 11,12-EET, and 14,15-EET also significantly increased ADPR production from NAD. Western blot analysis and immunoprecipitation demonstrated the presence of NAD glycohydrolase, which mediated 11,12-EET-induced activation of $K_{\text{Ca}}$ channels. HPLC analysis has shown that 11,12-EET increased the production of ADPR but not the formation of cADPR. In cell-attached patches, 11,12-EET (100 nM) increases $K_{\text{Ca}}$ channel activity by 5.6-fold. The NAD glycohydrolase inhibitor cibacron blue 3GA (3GA, 100 μM) significantly attenuated 11,12-EET-induced increase in the $K_{\text{Ca}}$ channel activity in CASMCs. However, 3GA had no effect on the $K_{\text{Ca}}$ channels activity in inside-out patches. 11,12-EET produced a concentration-dependent relaxation of precontracted coronary arteries. This 11,12-EET-induced vasodilation was substantially attenuated by 3GA and the ADP-ribosylation factor 3 (3GA, 100 μM) with maximal inhibition of 57%. These results indicate that 11,12-EET stimulates the production of ADPR and that intracellular ADPR is an important signaling molecule mediating 11,12-EET-induced activation of the $K_{\text{Ca}}$ channels in CASMCs and consequently results in vasodilation of coronary artery.

nicotinamide adenine dinucleotide glycohydrolase; K$^+$ channels; epoxyeicosatrienoic acid; endothelium-derived hyperpolarization factor

EPOXYEICOSATRIENOIC ACIDS (EETs), endothelium-derived arachidonic acid metabolites of cytochrome P-450, play an important role in the regulation of vascular tone (2, 13, 33, 48). In response to vasoactive substances such as acetylcholine, bradykinin, and arachidonic acid (AA), EETs are produced and released from endothelial cells of coronary, cerebral, and renal arteries (2, 31, 33, 48). EETs activate the Ca$^{2+}$-activated K$^+$ ($K_{\text{Ca}}$) channels, hyperpolarize vascular smooth muscle, and dilate vessels (9, 22, 23, 26, 31–33, 48), and therefore they are considered as endothelium-derived hyperpolarizing factors (2, 13). Recent studies in our laboratories and by others have shown that EETs induced activation of $K_{\text{Ca}}$ channels by several membrane-limited mechanisms such as the activation of Gs protein via ADP-ribosylation (3, 9, 23, 35). However, the activity of $K_{\text{Ca}}$ channels is also regulated by several different intracellular second messengers including cGMP and cAMP (8, 34). Although we demonstrated that adenylyl cyclase-cAMP and guanylyl cyclase-cGMP pathways are not involved in EETs-induced activation of $K_{\text{Ca}}$ channels (2, 23), our studies did not exclude the role of other cytoplasmic factors in mediating the action of EETs.

Recently, endogenous metabolites of NAD, cADP-ribose (cADPR) and ADP-ribose (ADPR), have been identified as intracellular signaling molecules (10, 12, 19, 20, 25, 42). cADPR is formed from NAD via ADP-ribosylation, and ADPR is produced by either hydrolysis of NAD via NAD glycohydrolase or hydrolysis of cADPR via cADPR hydrolase (14–16, 18, 25, 29). cADPR-mediated Ca$^{2+}$ signaling participates in the regulation of a variety of cell functions or cellular activities (11, 12, 18, 21, 24, 28, 38, 40, 43). However, the physiological role of ADPR as a signaling molecule remains unknown. Because ADP-ribosylation, a transfer process of ADPR to protein, has been demonstrated to mediate the effect of EETs on the $K_{\text{Ca}}$ channel activity (3, 23), it is possible that intracellular ADPR is also involved in the activation of $K_{\text{Ca}}$ channel by EETs. Indeed, ADPR may cause nonenzymatic ADP-ribosylation of proteins, which regulates a number of biological events, including DNA repair, translational regulation of cellular protein, platelet aggregation, and gating of the fertilization channel in ascidian oocytes (6, 15, 36, 41). More recently, we (27) have reported that ADPR activates $K_{\text{Ca}}$ channels in coronary arterial smooth muscle cells.
muscle cells. Thus ADPR-induced $K_{Ca}$ channel activation may contribute to the action of the EETs. The present study was designed to determine whether ADPR participates in 11,12-EETs-induced activation of the $K_{Ca}$ channels in CASMCs. First, we determined the biochemical pathways for ADPR production and the effect of 11,12-EET on these pathways. Then we directly determined the role of ADPR in 11,12-EET-induced activation of the $K_{Ca}$ channel using a patch-clamp technique and relaxation of coronary arteries by vascular reactivity studies.

**MATERIALS AND METHODS**

**Preparation of homogenate from small bovine coronary arteries.** Coronary arterial homogenates were prepared as we described previously (23). Briefly, bovine hearts were obtained from a local slaughterhouse. Small coronary arteries (250–300 μm) were microdissected under a dissecting stereomicroscope. These arteries were pooled and stored in ice-cold phosphate-buffered saline. 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) 25 Na-HEPES, 1 EDTA, 255 sucrose, and 0.1 phenylmethylsulfon fluoride. After centrifugation of the homogenized tissue at 6,000 g for 5 min at 4°C, the supernatant containing membrane and cytosolic components, termed homogenate, was aliquoted and frozen in liquid N$_2$ and stored at −80°C until use.

**Assay of NAD glycohydrolase in bovine coronary arterial homogenates.** To determine the activity of NAD glycohydrolase, the homogenates (50 μg) were incubated for 60 min with 1 mM NAD. All experiments were performed at 37°C in an assay buffer containing (in mmol/l) 250 potassium glutonate, 250 N-methylglucamine, 20 HEPES, and 1 MgCl$_2$ (pH 7.2). The conversion rate of NAD into ADPR represents the NAD glycohydrolase activity. To determine the activity of cADPR hydrolase, the conversion rate of cADPR into ADPR was measured after incubation of the sample with cADPR (0.5 mM) at 37°C for 30 min. The total reaction volume was 0.1 ml. The reaction mixture was then rapidly frozen in liquid N$_2$ to terminate the reaction. Before HPLC analysis, the reaction mixtures were centrifuged at 4°C using an Amicon microfiltration at 13,000 rpm for 10 min to remove the proteins. HPLC analysis was performed as described previously (27, 43). To determine the effects of 8,9-EET, 11,12-EET, and 14,15-EET on the activity of NAD glycohydrolase, the coronary homogenates were preincubated with the EETs, and then NAD was added and incubated for 60 min. AA and 20-hydroxyeicosatetraenoic acid, an AA P-450 o-hydrolase metabolite, (0.1 μmol/l), were used as a negative control.

**Western blot analysis.** Western blot was performed as described previously (22). Briefly, 30 μg of protein from the homogenates (microsomes or cytosols) were subjected to SDS-PAGE (12% running gel) after being heated at 100°C for 3 min. The protein was electrophoretically transferred onto a nitrocellulose membrane and then incubated with monoclonal antibody against human CD38 for 1 h at room temperature. CD38 possesses multiple enzyme activities including NAD glycohydrolase activity in a variety of tissues or cells (1, 4, 7, 14, 39, 45–47). After removal of the anti-CD38 antibody, the membrane was incubated for another 1 h with 1:1,000 horseradish peroxidase-labeled anti-mouse antibody. The detection solution 1 and 2 (1:1) (Amersham, IL) were added directly to the blots on the surface carrying the protein. After incubation for 1 min at room temperature, the membrane was wrapped in Saran Wrap and then exposed to Kodak Obst film.

**Immunoprecipitation.** Immunoprecipitation was performed as described previously (23). Briefly, the coronary arterial homogenate (140 μg) was incubated with the monoclonal antibody against CD38 (Pharmingen; San Diego, CA) for 18 h at 4°C. Samples were then incubated with protein A immobilized on Sepharose CL-4B beads (Sigma) for another 2 h at 4°C under constant rotation. Beads and supernatant were separated by centrifugation at 12,000 g for 5 min. Western blotting was used to confirm the removal of CD38. The supernatant was used to measure the activity of NAD glycohydrolase by HPLC.

**Patch-clamp study.** Smooth muscle cells were prepared, and the patch-clamp study was performed as we described previously (22). The bath solutions used for single channel recordings in the cell-attached mode contained (in mmol/l) 145 KCl, 1.8 CaCl$_2$, 1.1 MgCl$_2$, 10 glucose, and 5 HEPES (pH 7.4), and the pipette solution contained (in mmol/l) 145 KCl, 1.8 CaCl$_2$, 1.1 MgCl$_2$, and 5 HEPES (pH 7.4). The bath solution used for single channel recordings in the inside-out excised membrane patch contained (in mmol/l) 145 KCl, 1.1 MgCl$_2$, 10 HEPES, 2 EGTA, and 300 nmol/l ionized calcium (pH 7.2), and the pipette solution contained (in mmol/l) 145 KCl, 1.8 CaCl$_2$, 1.1 MgCl$_2$, and 10 HEPES, 10 (pH 7.4).

The effects of the specific NAD glycohydrolase inhibitor, cibacron 3GA (3GA, 1–100 μmol/l) (17), on the $K_{Ca}$ channel activity was determined in the presence or absence of 11,12-EET. After a cell-attached patch was established, a 3-min control recording was obtained at a membrane potential of +40 mV. The bath solution was then changed to contain 3GA (1–100 μmol/l), and a second successive 3-min recording at each concentration was obtained. To determine the effects of these inhibitors on 11,12-EETs-induced activation of the K$^+$ channels, patch-clamp recordings were performed in the cell-attached patch mode. A 3-min control recording was obtained at a membrane potential of +40 mV. The bath solution was then exchanged with the solution containing 11,12-EET (100 nmol/l) ($n=6$), and then a second successive 3-min recording was obtained. In another group of cells, 3GA (100 μmol/l) was added into the bath solution. A 3-min control recording was obtained at a membrane potential of +40 mV. The solution in the bath was then exchanged with the solution containing 11,12-EET (100 nmol/l), and then a second successive 3-min recording was obtained ($n=6$).

The inside-out patch mode was used to further determine the effects of 3GA on the activity of the K$^+$ channels. After inside-out patches were established, a 3-min control recording was obtained at a membrane potential of +40 mV ($n=5$). 3GA (100 μmol/l) was then added into the bath solution, and another 3-min control recording was obtained at the same membrane potential as above ($n=5$).

**Vascular reactivity studies.** Vascular reactivity in bovine coronary arteries was determined as we described previously (11, 44). Briefly, the epicardial left anterior descending coronary artery was dissected and placed in a Krebs bicarbonate solution. The rings were prepared and suspended in a 6-ml water-jacketed organ chamber at 37°C. The contractile responses were monitored using a computerized recording system. After an equilibration period of 1.5 h, the vessels were activated by addition of KCl (80 mmol/l) until reproducible contractions were obtained. One ring of each pair then received a vehicle (0.01% ethanol), and other rings received 3GA (30 μmol/l, $n=12$) for 15 min before the addition of the thromboxane mimetic U-46619 (20 nmol/l). After a sustained contraction by U-46619 was obtained, cumulative additions of 11,12 EET (10$^{-9}$ to 10$^{-5}$ M) were made every 4 min until...
a plateau response was reached. 11,12-EET-induced vasodilation was evaluated in the presence or absence of 3GA. To further determine the effect of inhibition of ADPR production on 11,12-EET-induced relaxation of resistance coronary arteries, these small coronary arteries (250–300 μm) were preconstricted by 50 ± 10% of their resting diameter with the thromboxane A2 analog U-46619. Once steady-state contraction was obtained, cumulative dose-response curves of 11,12-EET (0.1 nmol/l to 100 nmol/l) or sodium nitroprusside (SNP, 10 nmol/l to 100 nmol/l) were determined by measuring changes in the internal diameter. In another group of experiments, the arteries were preincubated with NAD glycohydrolase inhibitor 3GA (30 μmol/l), and the concentration-dependent response of the arteries to 11,12-EET or SNP were measured. During the whole experiments, physiological saline solution in the bath was continuously bubbled with a gas mixture of 95% O2-5% CO2 and maintained at 37 ± 0.1°C.

Statistical analysis. Data were presented as means ± SE. Significance of differences in mean values within and between multiple groups was examined using two-way ANOVA for repeated measures followed by a Duncan’s multiple range test. A Student’s t-test was used to examine significance of differences in means within and between groups.
RESULTS

Effect of 11,12-EET on the activity of NAD glycohydrolase. Figure 1 presents a representative reverse-phase HPLC chromatogram depicting the metabolism of NAD by coronary arterial homogenates. When the homogenates were incubated with NAD, products with retention times of 3.1 and 15.6 min coeluted with synthetic cADPR and ADPR, respectively (Fig. 1A). In the presence of 11,12-EET (100 nmol/l), ADPR production was markedly increased (Fig. 1B). The conversion rate of NAD into ADPR was increased from $2.54 \pm 0.2$ nmol min$^{-1}$ mg protein$^{-1}$ of control to $3.43 \pm 0.3$ nmol min$^{-1}$ mg protein$^{-1}$ in the presence of 100 nmol/l 11,12-EET. However, 11,12-EET had no effect on the production of cADPR (Fig. 1C).

To detect the effects of EET regioisomers, AA, and other AA metabolites on the NAD glycohydrolase, the coronary arterial homogenates were incubated with NAD in the presence of 8,9-EET, 11,12-EET, 14,15-EET, AA, or 20-HETE. As shown in Fig. 2, all three of the EETs significantly increased the conversion rate of ADPR. However, AA and 20-HETE had no effect on the synthesis of ADPR.

Effect of immunoprecipitation of CD38 on 11,12-EET-induced increase in the NAD glycohydrolase activity. Figure 3A presents a typical Western blot analysis of CD38 in coronary arteries. As indicated above, CD38 possesses NAD glycohydrolase activity. Two immunoreactive bands with molecular sizes of 42 and 90 kDa were recognized by a monoclonal antibody against CD38 in coronary arterial homogenates (Fig. 3A, lane H), microsomes (lane M), and cytosol (lane C). A puri-
fied calf spleen NAD glycohydrolase (lane N) and cell lysate from human white blood cells (lane W) were analyzed at same time as a positive control, and only one band with 42 kDa was recognized by this antibody. The identity of a 90-kDa immunoreactive band in bovine coronary arterial preparations was not clarified. Previous studies have reported that this band may be an oxidized dimer of CD38 (1). It is possible that we have detected this dimerized CD38 in coronary arteries, but not in purified CD38 or human white blood cells. The effect of removing CD38 from the homogenates of coronary arterial smooth muscle on NAD glycohydrolases activity was examined on the production of ADPR by HPLC analysis. 11,12-EET significantly increased the production of ADPR in coronary arterial rings. The maximal relaxation (92%) of 11,12-EET (100 nmol/l) occurred at 10 μmol/l 3GA). However, 3GA at 100 μmol/l markedly attenuated the 11,12-EET-induced increase in the production of ADPR. The conversion rate of NAD into ADPR was decreased from 4.42 ± 0.33 nmol·min⁻¹·mg protein⁻¹ in the presence of 100 μmol/l 3GA, a 84% reduction. However, 3GA had no effect on the activity of cADPR hydrolase (Fig. 4A). As shown in Fig. 4B, the production of ADPR was markedly increased in the presence of 11,12-EET (100 nmol/l). However, 3GA at 100 μmol/l significantly attenuated the 11,12-EET-induced increase in the production of ADPR. The conversion rate of NAD into ADPR was decreased from 4.42 ± 0.33 nmol·min⁻¹·mg protein⁻¹ in the presence of 100 nmol/l 11,12-EET to 2.6 ± 0.47 nmol·min⁻¹·mg protein⁻¹ after the addition of 3GA and 11,12-EET (Fig. 4B).

Effect of inhibition of NAD glycohydrolase on 11,12-EET-induced increase of KCa channel activity. Figure 5A presents typical recording of KCa channels in cell-attached patches, depicting the effect of NAD glycohydrolase inhibitor 3GA (100 μmol/l) on the 11,12-EET-induced activation of KCa channel. As in previous studies, 11,12-EET (100 nmol/l) increases the KCa channel activity by 5.6-fold (Fig. 5, A and C). 3GA alone decreases the activity of KCa channel in a concentration-dependent manner (Fig. 5B). In the presence of 3GA, the 11,12-EET-induced increases in opening of KCa channel were completely blocked. 3GA had no effect on KCa channels in inside-out patches (open probability = 0.043 ± 0.01 of control vs. 0.043 ± 0.01 with 100 μmol/l 3GA).

Effect of inhibition of NAD-glycohydrolase on 11,12-EET-induced relaxation of bovine coronary artery. Figure 6A shows that 11,12-EET produced a concentration-dependent relaxation in U-46619-precontracted coronary arterial rings. The maximal relaxation (92%) to 11,12-EET occurred at 10 μmol/l. Pretreatment of coronary arterial rings with NAD glycohydrolase inhibitor, 3GA (30 μmol/l) markedly attenuated 11,12-EET-induced relaxation by 41%. In microperfused and pressurized small coronary arteries, 11,12-EET was found to relax these resistance arteries even at 1 nmol/l (Fig. 6B). In the presence of 3GA, 11,12-EET-induced vasodilation was significantly decreased. However, 3GA had no effect on SNP-induced vasodilation (Fig. 6C).
DISCUSSION

NAD glycohydrolase converts NAD to ADPR (15–17, 29, 41, 44). Because ADPR increases K_{Ca} channel activity (27), we were interested in the regulation of the synthesis of ADPR and its signaling action in the coronary arterial smooth muscle. Using HPLC analysis, we found that homogenates from coronary arterial smooth muscle metabolized NAD to ADPR. The conversion rate of NAD to ADPR was significantly increased by 11,12-EET. Moreover, 8,9-EET, 11,12-EET, and 14,15-EET had similar stimulatory effects on the NAD glycohydrolase activity in the vascular smooth muscle. However, AA and 20-HETE, another cytochrome P-450 metabolite of AA, did not activate this enzyme. These results provide the first evidence indicating that NAD glycohydrolase may be an enzymatic target of EETs in coronary arterial smooth muscle and that EETs activate the NAD glycohydrolase to increase intracellular ADPR concentrations.

CD38 possesses NAD glycohydrolase activity in a variety of mammalian tissues or cells (1, 4, 7, 14, 39, 45–47). In the present study, CD38 was detected in coronary arteries by Western blot analysis. After removal of CD38 from coronary homogenates by immunoprecipitation, 11,12-EET-induced production of ADPR was significantly blocked. These results indicate that in bovine coronary arterial smooth muscle, 11,12-EET increases ADPR production through CD38-associated NAD glycohydrolase activity.

A selective inhibitor of NAD glycohydrolase 3GA attenuated basal activity of NAD glycohydrolase and also blocked 11,12-EET-induced increase of NAD glycohydrolase activity. These results further support the view that 11,12-EET-induced production of ADPR is due to the activation of NAD glycohydrolase. We performed patch-clamp experiments to examine the effect of 3GA on the activity of K_{Ca} channels in cell-attached patches. 3GA decreased the basal activity of K_{Ca} channels and completelyabolished 11,12-EET-induced activation of the K_{Ca} channel. However, in the inside-out patches, 3GA has no effect on the K_{Ca} channel. These results suggested that 11,12-EET-induced activation of K_{Ca} channels is through the activation of NAD glycohydrolase, which depends on the presence of cellular soluble substrate NAD. To further define the role of ADPR in 11,12-EET-induced vasorelaxation, vascular reactivity to 11,12-EET was examined in the absence or presence of 3GA. In the presence of 3GA, 11,12-EET-induced vasorelaxation was attenuated in both epicardial coronary arteries and small coronary arteries. However, 3GA had no effect on SNP-induced vasorelaxation. This suggests that ADPR may mediate 11,12-EET-induced activation of K_{Ca} channels in bovine coronary arterial smooth muscle and consequently result in vasorelaxation of these vessels. There is increasing evidence that NAD metabolites mediate the effects of a number of agonists in tissues or cells (45–47). In pancreatic β-cells, an ADP-ribosylcyclase product of NAD, cADPR mediates glucose-induced insulin secretion (38). cADPR may also mediate the effects of the activation of acetylcholine receptors in adrenal chromaffin cells, estrogen receptors in uterus, 5-hydroxytryptamine 2B receptors in arterial endothelial cells, and retinoic acid in renal tubular cells and aortic smooth muscle (5, 28, 37, 40). The present findings indicate that another NAD metabolite, ADPR may also serve as a signaling molecule, which mediates the effects of 11,12-EETs on coronary arterial smooth muscle. This role of ADPR in mediating the effect of EETs may represent a new signaling pathway regulating the activity of K_{Ca} channels and the action of endothelium-derived hyperpolarizing factors.

We have previously reported a role for G_{S} in mediating 11,12-EET-induced activation of the K_{Ca} channels (22). 11,12-EET stimulated the endogenous ADP-ribosylation of G_{S}, and the activation of G_{S} increased the activity of K_{Ca} channels. In other studies, EETs activated K_{Ca} channel through a G_{S}-mediated, membrane-delimited effect in HEK293 cells (9), which is consistent with our findings. The present study demonstrated that 11,12-EET activated NAD glycohydrolase, increased intracellular ADPR, and thereby induced activation of the K_{Ca} channels, resulting in the relaxation of coronary arteries. These results indicate that a cytoplasmic signaling nucleotide ADPR may mediate the EET effect. Therefore, the mechanisms mediating the action of EETs on K_{Ca} channel activity may be associated not only with ADP-ribosylation of G_{S}, but also with ADPR-mediated activation of these channels.

In summary, the present study demonstrates that NAD glycohydrolase present in bovine coronary arterial smooth muscle catalyzes the hydrolysis of NAD into ADPR. 11,12-EET activates K_{Ca} channels by increasing the production of ADPR through the activation of NAD glycohydrolase in coronary arterial smooth muscle. These results suggest that NAD glycohydrolase product, ADPR participates in the regulation of the activity of K_{Ca} channels in coronary vascular smooth muscle. ADPR may serve as intracellular second messenger mediating 11,12-EET-induced activation of the K_{Ca} channels. Therefore, ADPR may play a role in mediating endothelium-dependent hyperpolarization in the coronary circulation.

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REFERENCES

3. Campbell WB, Zou AP, and Li PL. Epoxyeicosatrienoic acid as endothelium-derived hyperpolarization factors in coronary arteries: potassium channels activation through endogenous ADP-ribosylation of Gs. In: Endothelium-Dependent Hyperpolariza-
ADP-ROBINE IN CORONARY ARTERY


Zocchi E, Franco L, Guida L, Benatti U, Bragellesi A, Malavasi F, Lee HC, and De Flora A. A single protein immunologically identified as CD38 displays NAD<sup>+</sup> glycohydrolase, ADP-riboseyl cyclase and cyclic ADP-ribose hydrolyase activities at...

