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Aldehyde dehydrogenase-independent bioactivation of nitroglycerin in porcine and bovine blood vessels

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ABSTRACT

The vascular bioactivation of the antianginal drug nitroglycerin (GTN), yielding 1,2-glycerol dinitrate and nitric oxide or a related activator of soluble guanylate cyclase, is catalyzed by aldehyde dehydrogenase-2 (ALDH2) in rodent and human blood vessels. The essential role of ALDH2 has been confirmed in many studies and is considered as general principle of GTN-induced vasodilation in mammals. However, this view is challenged by an early report showing that diphenyleneiodonium, which we recently characterized as potent ALDH2 inhibitor, has no effect on GTN-induced relaxation of bovine coronary arteries (De La Lande et al., 1996). We investigated this issue and found that inhibition of ALDH2 attenuates GTN-induced coronary vasodilation in isolated perfused rat hearts but has no effect on relaxation to GTN of bovine and porcine coronary arteries. This observation is explained by low levels of ALDH2 protein expression in bovine coronary arteries and several types of porcine blood vessels. ALDH2 mRNA expression and the rates of GTN denitration were similarly low, excluding a significant contribution of ALDH2 to the bioactivation of GTN in these vessels. Attempts to identify the responsible pathway with enzyme inhibitors did not provide conclusive evidence for the involvement of ALDH3A1, cytochrome P450, or GSH-S-transferase. Thus, the present manuscript describes a hitherto unrecognized pathway of GTN bioactivation in bovine and porcine blood vessels. If present in the human vasculature, this pathway might contribute to the therapeutic effects of organic nitrates that are not metabolized by ALDH2.

1. Introduction

The antianginal drug nitroglycerin (GTN) causes vasodilation through the activation of soluble guanylate cyclase (sGC), resulting in accumulation of 3′,5′-cyclic guanosine monophosphate (cGMP) in vascular smooth muscle. This effect is mediated by enzymatic release of nitric oxide (NO) or a related species, which binds with nanomolar affinity to the prosthetic heme group of sGC. The mechanism underlying bioactivation of GTN to yield NO has remained controversial for several decades. Besides the non-enzymatic reaction of GTN with cysteine [1,2] or ascorbate [3], several enzymes were proposed to catalyze GTN bioactivation, in particular GSH-S-transferase [4,5], cytochrome P450 [6,7], cytochrome P450 reductase [8] and xanthine oxidase, which exhibits nitrite reductase activity at low oxygen tension [9,10]. However, the involvement of xanthine oxidase-catalyzed nitrite reduction was later excluded [11], and neither of the other pathways appears to fully explain GTN vasoactivity [12].

In 2002, Stamler and coworkers proposed aldehyde dehydrogenase-2 (ALDH2) as the key enzyme that catalyzes GTN bioactivation [13], and meanwhile there is general agreement that this pathway is essentially involved in GTN-induced relaxation in rodent and

Abbreviations: ALDH, aldehyde dehydrogenase; CB25, 1-{[4-(1,3-benzodioxol-5-ylmethyl)-1-piperazinyl]methyl}-1H-indole-2,3-dione; cGMP, 3′,5′-cyclic guanosine monophosphate; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; DMSO, dimethyl sulfoxide; DPI, diphenyleneiodonium; DTPA, diethylenetriamine pentaacetic acid; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; GDN, glycerol dinitrate; GTN, glycerol trinitrate (nitroglycerin); NAD, nicotinamide adenine dinucleotide; L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one; sGC, soluble guanylate cyclase..

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human blood vessels [14]. The evidence is based on inhibition of GTN-induced relaxation by ALDH2 inhibitors [13] and the absence of the high affinity pathway of GTN vasodilation in ALDH2-deficient mice [15]. The main route of ALDH2-catalyzed denitration of GTN yields 1,2-glycerol dinitrate (1,2-GDN) and inorganic nitrite, but our data obtained with several ALDH2 mutants suggest that GTN bioactivity is mediated by a minor pathway resulting in the direct formation of NO [16].

The involvement of ALDH2 in GTN-induced relaxation has been demonstrated for rabbit [13], rat [17–19], mouse [15,20], guinea pig [21], and human [22,23] blood vessels, suggesting a general principle of vascular GTN bioactivation in mammals. However, in an early study Horowitz and coworkers showed that diphenyleneiodonium (DPI), which we recently characterized as potent ALDH2 inhibitor [24], does not affect relaxation to GTN of bovine coronary arteries [25]. In the present study we confirm this observation, extend it to porcine coronary arteries, and show that ALDH2 expression is very low in bovine coronary arteries and several types of porcine vessels. The results point to an ALDH2-independent pathway of GTN bioactivation that may be relevant for the pharmacology of organic nitrates, in particular the isosorbide nitrates, which are not metabolized by ALDH2.

2. Materials and methods

2.1. Materials

Human ALDH2 was expressed in *Escherichia coli* BL21 (DE3) and purified as described previously [26,27]. Ethylenediamine tetraacetic acid-(EDTA)-free Complete™ Protease Inhibitor Cocktail Tablets were from Roche Diagnostics GmbH (Vienna, Austria). [2-^{14}C]GTN (50 mCi/mmol) was from American Radiolabeled Compounds, purchased through Hartmann Analytic GmbH (Braunschweig, Germany). Nitro POHL® ampoules (G. Pohl-Boskamp GmbH & Co., Hohenlockstedt, Germany), containing 4.4 mM GTN in 250 mM glucose, were obtained from a local pharmacy and diluted with distilled water. Unlabelled organic nitrates used as standards in radio thin layer chromatography (GTN, 1,2-GDN and 1,3-GDN) were purchased from LGC Standards (Wesel, Germany). 2,2-Diethyl-1-nitroso-oxyhydrazine (DEA/NO) and 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) were from Enzo Life Sciences (Lausen, Switzerland) purchased through Eubio (Vienna, Austria). DEA/NO was dissolved and diluted in 10 mM NaOH. The selective ALDH3A1 inhibitor 1-{[4-(1,3-benzodioxol-5-ylmethyl)-1-piperazinyl]methyl}-1H-indole-2,3-dione (CB25) [28] was obtained from ChemBridge Corporation (San Diego, CA, USA). All other chemicals were from Sigma–Aldrich (Vienna, Austria), including 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α (U-46619), DPI, N^G-Nitro-L-arginine (L-NNA) and chloral hydrate. Stock solutions of ODQ (100 mM), DPI (10 mM) and U-46619 (0.1 mM) were prepared in dimethyl sulfoxide or ethanol and further diluted in buffer. Final concentration of organic solvents did not exceed 0.1%.

2.2. Animals and tissues

Sprague-Dawley rats (obtained from Charles River, Sulzfeld, Germany) of either sex were housed at the local animal facility in approved cages and kept on a regular 12-hour dark/light cycle. They were fed standard chow (Altromin 3023; obtained from Königshofer Futtermittel (Ebergassing, Austria)) and received water *ad libitum*. Animals were euthanized in a box that was gradually filled with CO_2 until no more vital signs (cessation of respiration and circulation) were noticed. Subsequently, the thorax of the animals was opened. The thoracic aorta (and for some experiments heart and liver) were removed and placed in chilled buffer.

Porcine and bovine hearts were obtained from a local abattoir and immediately transported to the laboratory. The right coronary artery was carefully explanted and immediately used for assessment of vessel function.

2.3. Ring experiments (organ bath)

For isometric tension measurements, isolated blood vessels were cut into rings of ~3 mm length, and the rings suspended in 5-ml organ baths, containing oxygenated Krebs–Henseleit buffer (concentrations in mM: NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2, D-glucose 10.1; pH 7.4) as previously described in detail [24]. After equilibration for 60 min at the optimal resting tension (rat aorta: 1 g; porcine coronary artery: 2 g; bovine coronary artery: 4 g), maximal contractile activity was determined with a depolarizing solution containing 100 mM K^+. Rings that did not elicit adequate and stable contraction to high K^+ were considered as being damaged and omitted from the study. After washout, tissues were precontracted with the thromboxane mimetic U-46619. Addition of 50 nM U-46619 contracted rat aortas to ~90% of contraction obtained with 100 mM K^+, while 50 nM U-46619 contracted porcine and bovine coronary arteries to ~60% of maximal contraction. If indicated, chloral hydrate (1 mM), DPI (0.3 μM), L-NNA (1 mM) or ODQ (100 μM) were added simultaneously with U-46619 to the preparation. After a stable tone had been reached (~20 min), cumulative concentration–response curves were established in separate rings with GTN (0.1 nM–300 μM) or DEA/NO (1 nM–10 μM). The contractile force corresponding to each agonist concentration was recorded and expressed as percent of precontraction. For comparison of agonist potency in aortas and coronary arteries, concentration–response curves to GTN and DEA/NO were additionally established with rat aortic rings precontracted with 20 nM instead of 50 nM U-46619 (yielding ~50% of maximal contraction; see Legend to Fig. 2).

2.4. Measurement of coronary flow in perfused isolated rat hearts

Hearts from euthanized animals were mounted on a heart perfusion apparatus (Hugo Sachs Elektronik/Harvard Instruments, March-Hugstetten, Germany) and retrogradely perfused at 37 °C with oxygenated Krebs–Henseleit buffer at 80 mm Hg (constant pressure perfusion) as previously described [21]. The following cardiac parameters were monitored in unpaced hearts: coronary flow (as index of coronary arterial function) with a transonic flow probe, left-ventricular developed pressure *via* a fluid-filled balloon that was inserted into the left ventricle and connected to a pressure transducer, and heart rate, derived electronically from the pressure signal.

After equilibration for 30 min (baseline), coronary relaxation was induced with GTN given as bolus injections through a sideline in non-cumulative manner, resulting in final concentrations of ~1 nM to 100 μM (5 min per dose). After the last dose, GTN was washed out for 30 min, and baseline was re-established. Thereafter, a concentration–response curve to DEA/NO (1 nM–10 μM) was established (total duration of the experiment 120 min). To test for the involvement of ALDH2, experiments were performed in the absence and presence of 0.1 μM DPI, added to the perfusion buffer.

2.5. Immunoblotting

Freshly isolated aortas or coronary arteries were cleaned, weighed and pre-digested with collagenase (1.5 mg/ml) in 10 mM Tris-buffer, pH 7.4, containing 250 mM sucrose, 3 mM $CaCl_2$,