

Is the mechanism of nitroglycerin tolerance associated with aldehyde dehydrogenase activity? A contribution to the ongoing discussion*

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The aim of the study presented here was an attempt to answer the question posed in the title: Is the mechanism of nitroglycerin tolerance associated with aldehyde dehydrogenase (ALDH) activity? Here, we investigated the effect of administration (separately or jointly) of lipoic acid (LA), nitroglycerin (GTN), and disulfiram (DSF; an irreversible *in vivo* inhibitor of all ALDH isozymes (including ALDH2)), on the development of tolerance to GTN. We also assessed the total activity of ALDH in the rat liver homogenates. Our data revealed that not only DSF and GTN inhibited the total ALDH activity in the rat liver, but LA also proved to be an inhibitor of this enzyme. At the same time, the obtained results demonstrated that the GTN tolerance did not develop in GTN, DSF and LA jointly treated rats, but did develop in GTN and DSF jointly treated rats. This means that the ability of LA to prevent GTN tolerance is not associated with the total ALDH activity in the rat liver. In this context, the fact that animals jointly receiving GTN and DSF developed tolerance to GTN, and in animals that in addition to GTN and DSF also received LA such tolerance did not develop, is – in our opinion – a sufficient premise to conclude that the nitrate tolerance certainly is not caused by a decrease in the activity of any of the ALDH isoenzymes present in the rat liver, including ALDH2. However, many questions still await an answer, including the basic one: What is the mechanism of tolerance to nitroglycerin?

Key words: nitroglycerin tolerance, aldehyde dehydrogenase, disulfiram, lipoic acid

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Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; cGMP, cyclic GMP; DHLA, dihydrolipoic acid; DTT, dithiothreitol; DSF, disulfiram; 1,2-GDN, 1,2-glyceryl dinitrate; GTN, glyceryl trinitrate, nitroglycerin; LA, lipoic acid; NO, nitric oxide; sGC, soluble guanylate cyclase; WBCs, white blood cells; XO, xanthine oxidase

INTRODUCTION

Since 1879, when Murrell discovered the antianginal effect of a 1% solution of oral nitroglycerin (glyceryl trinitrate; GTN), it became the most popular drug in everyday medical practice. It is used in the ambulance, outpatient and inpatient care. Its usefulness in ending sudden angina in documented coronary artery disease is unquestioned (Kosmulski *et al.*, 2019).

GTN is thought to be converted in the vasculature into nitric oxide (NO) or some NO adjunct which activates the soluble guanylate cyclase (sGC) and subsequently elevates the cGMP level, resulting in vasodilatation (Murad, 2004). However, long-term GTN therapy causes development of nitrate tolerance, which is defined as a reduced response to treatment with organic nitrates, or the need to increase dosages to maintain the effects. PubMed lists more than 15000 publications on GTN. It is worth to recall that the first work describing the phenomenon of GTN tolerance was published in 1898 (Laws, 1898). However, the molecular mechanism of GTN biotransformation has remained a mystery and it is not well understood why tolerance manifests over time.

Aldehyde dehydrogenase (ALDH, EC 1.2.1.3) is a polymorphic enzyme responsible for many life processes (cell division, differentiation, proliferation, response to oxidative stress and cellular homeostasis) and oxidation of endogenous and exogenous aldehydes to their respective carboxylic acids (Christy & Doss, 2014; Vasiliou *et al.*, 2000). In mammals, the ALDH isozymes are traditionally divided into three classes: class 1 (ALDH1; cytosolic), class 2 (ALDH2; mitochondrial), and class 3 (ALDH3; such as those expressed in tumors, stomach, and cornea). ALDH1 and ALDH2 are the most important enzymes for aldehyde oxidation. These enzymes are found in many tissues of the body, but occur in the liver at the highest concentration. Mammalian liver class 1 and class 2 ALDH isoforms were purified in the 1970s, and have been used extensively as models for testing their kinetic and structural properties. It is now known that the kinetic properties and subcellular distribution of the major mitochondrial and cytosolic ALDH isozymes isolated from different species are similar. Mitochondrial (low- K_m) ALDH2 is the major enzyme oxidizing ethanol-derived acetaldehyde, both in humans and rats (Moon *et al.*, 2007).

Taken together, in rodents, both ALDH1 and ALDH2 are involved in the acetaldehyde metabolism because ALDH1 isozymes also exhibit a relatively low K_m values (11–18 μM range) for acetaldehyde; in humans, ALDH2

plays a major role in acetaldehyde detoxification following ethanol metabolism *in vivo*, since it has a very low K_m value ($\leq 1.0 \mu\text{M}$ range) for acetaldehyde (Klyosov *et al.*, 1996).

In 2002, Chen and others (Chen *et al.*, 2002) demonstrated *in vitro* and *in vivo* that ALDH2 catalyzes formation of 1,2-glyceryl dinitrate (1,2-GDN) and nitrite from low concentrations of GTN. The reaction required DTT (dithiothreitol) or 2-mercaptoethanol as reductants, and was stimulated by the ALDH cofactor NAD. These studies have also indicated that during this reaction ALDH2 is oxidatively inhibited by GTN, causing down-regulation of this enzyme which is associated with GTN tolerance (Chen *et al.*, 2002). By using an *in vivo* model of nitrate tolerance, DiFabio and others (DiFabio *et al.*, 2003) found that inhibitors of ALDH2 caused a similar shift in the relaxation responses of tolerant and nontolerant aortas. In 2005, Kollau *et al.* indicated that ALDH2 contributed to GTN bioactivation in the blood vessels and rat liver mitochondria, but it was not the only mammalian enzyme catalyzing this process (Kollau *et al.*, 2005).

In 2007, Wenzel and others (Wenzel *et al.*, 2007) reported that the activity of ALDH2 (oxidatively inhibited by GTN) was restored *in vitro* by dithiol compounds, such as the dithiothreitol (DTT) and dihydrolipoic acid (DHLA, 6,8-dimercapto-octanoic acid) (Wenzel *et al.*, 2007). On the other hand, *in vivo* experiments indicated that treatment of rats with lipoic acid (LA, 1,2-dithiolano-3-pentanoic acid) in combination with and GTN, did not provide any protection against GTN-induced ALDH inhibition (it is worth mentioning that DHLA is formed in the body by LA reduction; Bilka-Wilkosz *et al.*, 2016).

Beretta and others (Beretta *et al.*, 2008) demonstrated *in vitro* that not only ALDH2, but also ALDH1 could convert GTN into significant amounts of NO. In 2011, experiments in the rat liver homogenates confirmed that the cytoplasmic isoform, i.e. ALDH1, was also capable of reducing GTN to biologically active NO (Tsou *et al.*, 2011). Axton and others (Axton *et al.*, 2018) indicated that xanthine oxidase (XO; EC 1.17.3.2) was a critical enzyme for metabolic bio-activation of GTN to NO.

The discourse continues, and these problems have not been resolved yet.

Thus, as pointed out in the title, the study presented here is a modest contribution to the almost two decades-long discussion on this topic. The aim of this study is an attempt to answer the question: Is the mechanism of nitroglycerin tolerance associated with ALDH activity? Here, we investigated the effect of administering LA, GTN and an inhibitor of all ALDH isozymes - disulfiram (DSF), on the development of tolerance to GTN. We also assessed the total activity of ALDH in the rat liver homogenates.

MATERIALS AND METHODS

Animals. All procedures were performed according to the Animal Use and Care Committee Guidelines and were approved by the Ethical Committee on Animal Testing at the Jagiellonian University, Kraków, Poland (registration number 106/2009 and ZI/UJ/403/2007).

The experiments were carried out on male Wistar rats (180–250 g). Animals were housed in a room in plastic cages at a constant temperature of $20 \pm 2^\circ\text{C}$, with natural light–dark cycle. They had free access to standard pellet diet and water.

In experiment A, we measured the systolic and diastolic blood pressure in all animals before GTN administration into the caudal vein, and during 20 min thereafter. GTN tolerance was confirmed when GTN administered on the 5th day did not decrease the arterial blood pressure vs. control arterial blood pressure measured before GTN administration *in vivo*. The animals were randomly divided into seven groups of 8 animals each. The groups were treated as follows:

group 1: 0.9% NaCl, *sc*, 0.6 ml, for 4 days, on the 5th day GTN at a dose of $150 \mu\text{g}/\text{kg}$, *iv* (control group);

group 2: DSF at a dose of $150 \text{mg}/\text{kg}$, *ip*, daily divided into two doses, for 4 days, on the 5th day GTN at a dose of $150 \mu\text{g}/\text{kg}$, *iv*;

group 3: GTN at a dose of $30 \text{mg}/\text{kg}$, *sc*, daily divided into three doses, for 4 days, on the 5th day GTN at a dose of $150 \mu\text{g}/\text{kg}$, *iv*;

group 4: GTN at a dose of $30 \text{mg}/\text{kg}$, *sc*, daily divided into three doses, for 4 days + DSF for 4 days, a dose of $150 \text{mg}/\text{kg}$, *ip*, divided into two doses, on the 5th day GTN at a dose of $150 \mu\text{g}/\text{kg}$, *iv* (GTN-tolerance group);

group 5: LA at a dose of $100 \text{mg}/\text{kg}$, *ip*, divided into two doses, for 4 days + DSF for 4 days at a dose of $150 \text{mg}/\text{kg}$, *ip*, divided into two doses, on the 5th day GTN at a dose of $150 \mu\text{g}/\text{kg}$, *iv*;

group 6: GTN at a dose of $30 \text{mg}/\text{kg}$, *sc*, daily divided into three doses for 4 days + DSF for 4 days at a dose of $150 \text{mg}/\text{kg}$, *ip*, divided into two doses + LA for 4 days at a dose of $100 \text{mg}/\text{kg}$, *ip*, divided into two doses, on the 5th day GTN at a dose of $150 \mu\text{g}/\text{kg}$, *iv*;

group 7: LA at a dose of $100 \text{mg}/\text{kg}$, *ip*, daily divided into two doses, for 4 days, on the 5th day GTN at a dose of $150 \mu\text{g}/\text{kg}$, *iv*.

Blood pressure measurement. The rats were anesthetized with thiopental ($70 \text{mg}/\text{kg}$) *ip* injection. The left carotid artery was cannulated with polyethylene tubing filled with a heparin solution in saline to facilitate pressure measurements using the Datamax apparatus (Columbus Instruments, Ohio). Blood pressure was measured before GTN ($150 \mu\text{g}/\text{kg}$; *iv*) administration into the caudal vein and during 20 min thereafter. Subsequently, animals were sacrificed by cervical dislocation and the livers were excised, washed in 0.9% NaCl, placed in liquid nitrogen and stored at -80°C until the ALDH activity test was performed.

Aldehyde dehydrogenase activity. The frozen livers were weighed and homogenates were prepared by homogenization of 1 g of the tissue in 4 ml of 0.1 M phosphate buffer, pH 7.4, using an IKA-ULTRA-TURRAX T8 homogenizer. Total aldehyde dehydrogenase (ALDH) activity was measured as NADH formation from NAD^+ in the presence of propionaldehyde by monitoring an increase in the absorbance at 340 nm, using a modified protocol published earlier (Loomis & Brien, 1983; Tottmar *et al.*, 1973).

The assay mixture (1 ml) contained 50 mM sodium pyrophosphate buffer (pH 8.2), 1 mM NAD, 1 mM EDTA, 1 mM 4-methylpyrazole (to inhibit alcohol dehydrogenase, ADH), $2 \mu\text{M}$ rotenone (to inhibit NADH consumption by complex I of the electron transfer chain). The reaction was initiated by addition of 10 mM propionaldehyde to the cuvette, and absorbance change was monitored for 3 min at 37°C to calculate the rate of NADH production.

ALDH activity was calculated by using the molar extinction coefficient of reduced NAD(P) of $6.22 \text{mM}^{-1}\text{cm}^{-1}$ at 340 nm. Enzyme-specific activity was expressed as $\text{nmol NADH min}^{-1} \text{mg}^{-1} \text{protein}$.

Protein content. Protein content was assayed by the Lowry and others (Lowry *et al.*, 1951) method which is based on the reaction of peptide bonds and aromatic amino acid residues of proteins with the Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolibdenic acid) in an alkaline environment, in the presence of cupric ions. Copper (II) ions bound to the protein tyrosine and tryptophan residues reduce the above acids to oxides.

Absorbance was measured at 500 nm. A 1% solution of bovine albumin was used to prepare a standard curve.

Drugs and compounds. Nitroglycerin was purchased from LEK S.A. and Pliva Kraków S.A. (Poland). Lipoic acid was a gift from Hexal® AG, (Holzkirchen, Germany). Disulfiram was purchased from Sigma-Aldrich Chemical Company (Poznań, Poland). Thiopental sodium was obtained from HEFA-Frenon Arzneimittel (Germany). The Folin-Ciocalteu reagent, β -nicotinamide adenine dinucleotide tetrasodium salt (NAD), EDTA, rotenone, propionaldehyde, and 4-methylpyrazole were provided by Sigma-Aldrich Chemical Company (Poznań, Poland). All the other reagents were of analytical grade and were obtained from Polish Chemical Reagent Company (POCH, Gliwice, Poland).

Statistical analysis. All statistical calculations were carried out with the STATISTICA 8.0 computer program. The mean and standard deviation of the mean were calculated for each group. Normal distribution was verified with the Kolmogorow-Smirnow test. Data were analyzed by the Student's *t*-test. Differences were considered as statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Our data revealed that not only DSF and GTN inhibited the total ALDH activity in the rat liver, but LA also proved to be an inhibitor of this enzyme (Table 1). As for DSF, it is obvious. In case of GTN, this is confirmed by literature data. GTN-induced inhibition of the ALDH activity was already noticed by Towell and others (Towell *et al.*, 1985) in 1985. Notably, this was done with ALDH found in erythrocytes, which is a cytoplasmic isoform of this enzyme. It is also known that GTN potently inhibited both, purified yeast ALDH and GTN-treated rats' ALDH2 from isolated cardiac mitochondria *in vivo* (Wenzel *et al.*, 2007). It was also demonstrated that GTN inhibited ALDH2 activity in circulating white blood cells (WBC) of healthy volunteers (Wenzel *et al.*, 2009). Beretta and others (Beretta *et al.*, 2008) showed

that GTN blocked ALDH2 activity in isolated rat liver mitochondria, as well as activity of recombinant human ALDH2.

At the same time, the study presented here demonstrated that DSF treatment alone (without GTN) and GTN treatment alone (without DSF) did not cause tolerance after 4 days of GTN administration, since the animals responded to intravenous administration of GTN on day 5 with a significant drop in blood pressure (Fig. I-2 and I-3, respectively). However, the obtained results showed that tolerance developed in the group jointly treated with GTN and DSF, in which intravenous administration of GTN on day 5 did not elicit any significant drop in blood pressure (Fig. I-4). Our earlier studies demonstrated that GTN treatment alone (without DSF) induced tolerance after 8 days (Dudek *et al.*, 2008). Results presented here also demonstrate that DSF accelerated GTN tolerance in rats, but it did not cause tolerance *per se*, even though it inhibited the ALDH activity. At the same time, our data revealed that tolerance did not develop in animals jointly treated with GTN, DSF and LA, although LA was not only unable to restore the ALDH activity inhibited by DSF, but on the contrary, it acted as an inhibitor of this enzyme (Fig. I-6; Table 1). This is confirmed by our earlier studies which concentrated on the protective effect of LA in rats with acute intoxication due to the combined administration of ethanol and DSF (Bilska-Wilkosz *et al.*, 2019b). Taken together, our data indicate that the ability of LA to prevent GTN tolerance is not associated with ALDH activity in the rat liver.

Results obtained by many researchers seem to support the thesis that tolerance to GTN is not affiliated with the activity of ALDH2. Li and others (Li *et al.*, 2006) investigated the relationship between interindividual variability in the efficacy of sublingual GTN and ALDH2 polymorphism in a Chinese population. The studies showed that among the population showing normal ALDH-2 – genotype (Glu504 homozygotes), GTN was efficacious in 85.1% persons, while among those carrying ALDH-2*1/2 (Glu504Lys heterozygotes) or ALDH-2*2/2 (Lys504 homozygotes) genotype, only 57% reacted in this way. The authors suggested that history of alcohol intolerance can be a simple marker of a potential GTN tolerance (Li *et al.*, 2006). However, these results can be discerned in a different way, namely: why did 7 persons (14.9%) with normal ALDH-2 activity react to GTN abnormally and why did 19 persons (42.4%) with genetically determined low activity of ALDH-2 react to GTN normally? Moreover, a literature survey failed to find a reference documenting altered therapeutic effects of nitrates in an Asian population. Chen *et al.* showed that ALDH-2-deficient mice (ALDH-2^{-/-}) did not develop GTN tolerance at substantially higher concentrations of GTN (Chen *et al.*, 2005). Mayer and Beretta (Mayer & Beretta, 2008) had stressed that the involvement of ALDH-2 inactivation in vascular tolerance to GTN was controversial.

In vitro studies with the use of human ALDH2 and isolated animal tissues (blood vessels, liver and heart) provide evidence for an efficient and potent ALDH2-independent pathway of GTN bio-activation in the porcine and bovine coronary arteries. The authors suggested that if this pathway was present in human blood vessels, it might contribute to the therapeutic effect of organic nitrates that are not metabolized by ALDH2 (Neubauer *et al.*, 2015). A recent study by D'Souza *et al.* demonstrated that during development of GTN tolerance in rats and during tolerance reversal, changes in the GTN-

Table 1. The effect of various combinations of disulfiram (DSF), nitroglycerin (GTN), and lipoic acid (LA) on the total activity of aldehyde dehydrogenase (ALDH) in the rat liver.

Treatment	Activity of ALDH in the rat liver (mIU/mg protein)
Control (group 1)	46.09±9.90
DSF (group 2)	24.30 ^{aaa} ±7.70
GTN (group 3)	29.00 ^{aaa} ±8.45
GTN + DSF (group 4; tolerant group)	23.48 ^{aaa} ±4.37
LA and DSF (group 5)	27.40 ^{aaa} ±5.15
GTN + DSF + LA (group 6)	23.60 ^{aaa} ±6.64
LA (group 7)	30.59 ^{aaa} ±1.44

Data are shown as the mean ± standard deviation (S.D.) of 8 animals per group; ^{aaa} $p < 0.001$ vs. group receiving 0.9% NaCl (control group).

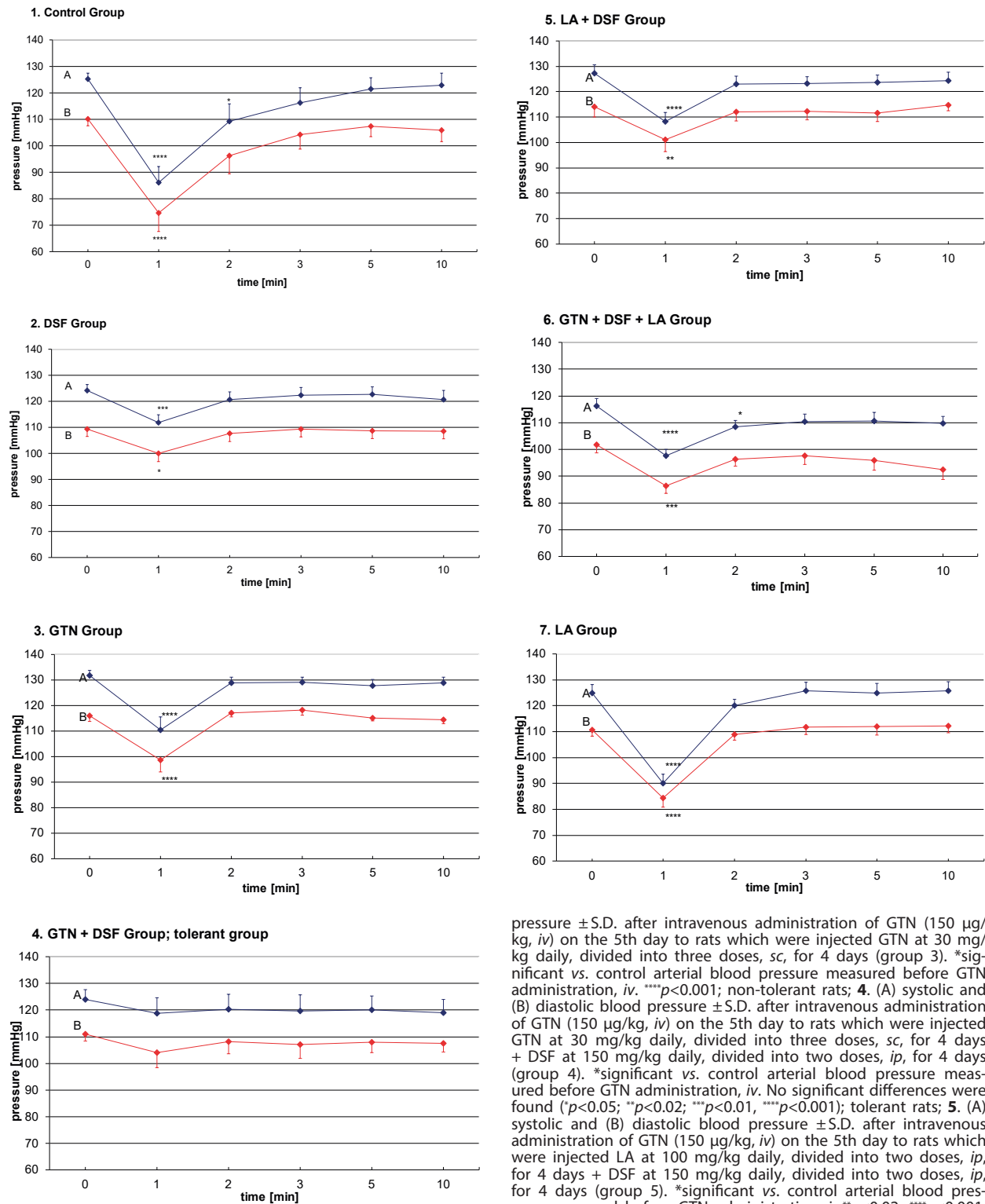


Figure I. The effect of various combinations of disulfiram (DSF), nitroglycerin (GTN), and lipoic acid (LA) on the systolic (A; blue line) and diastolic (B; red line) blood pressure in rats.

1. (A) systolic and (B) diastolic blood pressure \pm S.D. after intravenous administration of GTN (150 μ g/kg, *iv*) on the 5th day to rats which received 0.6 ml 0.9%NaCl, *sc*, for 4 days (group 1 – control group). *significant vs. control arterial blood pressure measured before GTN administration, *iv*. **** p <0.01; **** p <0.001; non-tolerant rats; **2.** (A) systolic and (B) diastolic blood pressure \pm S.D. after intravenous administration of GTN (150 μ g/kg, *iv*) on the 5th day to rats which were injected DSF at 150 mg/kg daily, divided into two doses, *sc*, for 4 days (group 2). *significant vs. control arterial blood pressure measured before GTN administration, *iv*. * p <0.05; *** p <0.01; non-tolerant rats; **3.** (A) systolic and (B) diastolic blood

pressure \pm S.D. after intravenous administration of GTN (150 μ g/kg, *iv*) on the 5th day to rats which were injected GTN at 30 mg/kg daily, divided into three doses, *sc*, for 4 days (group 3). *significant vs. control arterial blood pressure measured before GTN administration, *iv*. **** p <0.001; non-tolerant rats; **4.** (A) systolic and (B) diastolic blood pressure \pm S.D. after intravenous administration of GTN (150 μ g/kg, *iv*) on the 5th day to rats which were injected GTN at 30 mg/kg daily, divided into three doses, *sc*, for 4 days + DSF at 150 mg/kg daily, divided into two doses, *ip*, for 4 days (group 4). *significant vs. control arterial blood pressure measured before GTN administration, *iv*. No significant differences were found (* p <0.05; ** p <0.02; *** p <0.01, **** p <0.001); tolerant rats; **5.** (A) systolic and (B) diastolic blood pressure \pm S.D. after intravenous administration of GTN (150 μ g/kg, *iv*) on the 5th day to rats which were injected LA at 100 mg/kg daily, divided into two doses, *ip*, for 4 days + DSF at 150 mg/kg daily, divided into two doses, *ip*, for 4 days (group 5). *significant vs. control arterial blood pressure measured before GTN administration, *iv*. * p <0.02; **** p <0.001; non-tolerant rats; **6.** (A) systolic and (B) diastolic blood pressure \pm S.D. after intravenous administration of GTN (150 μ g/kg, *iv*) on the 5th day to rats which were injected GTN at 30 mg/kg daily, divided into three doses, *sc*, for 4 days + LA at 100 mg/kg daily, divided into two doses, *ip*, for 4 days (group 6). *significant vs. control arterial blood pressure measured before GTN administration, *iv*. *** p <0.01; **** p <0.001; non-tolerant rats.

mediated vasodilator responses of several types of blood vessels (aorta, vena cava, femoral artery and femoral vein) and in vascular GTN biotransformation did not correlate with changes in the ALDH activity or ALDH2 protein expression. The authors suggested that factors other than impaired ALDH2-mediated GTN bio-activation contributed to the GTN tolerance (D'Souza *et al.*, 2011).

It seems necessary to add at this point that our studies regarding ALDH activity were conducted on rat liver homogenates, prepared in such a way that mitochondria were not destroyed during their preparation. It means that we measured the total ALDH activity in these samples. Is this fact such a serious limitation of this research that the conclusions are unreliable? We hope that it is not. We designed our research to achieve the intended goal in as straightforward way as possible. That is why we decided to use the inhibitor of all ALDH isozymes, i.e. DSF, whose action is irreversible *in vivo*, and restoration of activity of all ALDH isozymes (including ALDH2), is only dependent on *de novo* protein synthesis, in contrast to *in vitro* conditions, where the mechanism of action of this inhibitor is completely different from the one *in vivo* (Bilska-Wilkosz *et al.*, 2019a; Kitson, 1975; Shen *et al.*, 2000; Vallari & Pietruszko, 1982).

Therefore, we conducted *in vivo* studies assuming that if ALDH is involved in GTN metabolism, tolerance should develop earlier in rats treated with GTN and DSF in combination, when compared to GTN – only treated animals (without DSF). We also expected tolerance to develop in animals treated with GTN, DSF and LA in combination, since there is currently no drug that would restore the activity of all ALDH isozymes *in vivo*, including ALDH2, when inhibited by DSF. In this case, however, there was no tolerance. Thus, LA is not able to restore the activity of any of the ALDH isoenzymes, including ALDH2, and yet counteracts the phenomenon of tolerance. This means that the anti-tolerance activity of LA is not associated with any ALDH isoenzyme, including ALDH2.

Finally, the fact that animals jointly receiving GTN and DSF developed tolerance to GTN, and animals that in addition to GTN and DSF also received LA did not develop such a tolerance, in our opinion is a sufficient premise to conclude that the nitrate tolerance certainly is not caused by a decrease in the activity of any of the ALDH isoenzymes present in the rat liver, including ALDH2. However, many questions still await an answer, including this basic one: What is the mechanism of tolerance to nitroglycerin?

Therefore, the discourse still continues.

Conflict of Interest

The authors do not have any conflict of interest regarding this manuscript.

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