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The mitochondrial uncoupler 2,4-dinitrophenol attenuates sodium nitroprusside-induced toxicity in *Drosophila melanogaster*: Potential involvement of free radicals

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ABSTRACT

The toxicity of sodium nitroprusside (SNP) (an inducer of oxidative/nitrosative stress) and the attenuation of SNP effects by 2,4-dinitrophenol (DNP) (that induces mild uncoupling of respiration) were evaluated in the *Drosophila melanogaster* model system. Fly larvae were raised on food supplemented with 1.0 mM SNP, 0.5 or 1.25 mM DNP, or with mixtures 1.0 mM SNP plus 0.5 or 1.25 mM DNP. Food supplementation with SNP decreased larval viability and pupation height whereas supplementation with DNP substantially reversed these changes. Biochemical analyses of oxidative stress markers and activities of antioxidant and associated enzymes were carried out on 2-day-old flies emerged from control larvae and larvae fed on food supplemented with SNP, DNP, or SNP/DNP mixtures. Larval exposure to SNP lowered activities of aconitase, while the presence of DNP reduced the negative impact of SNP by raising aconitase activity back to near control levels. Larval treatment with SNP also elevated the contents of carbonyl protein, uric acid and low molecular mass thiols and produced higher activities of superoxide dismutase, glutathione S-transferase, glucose-6-phosphate dehydrogenase and thioredoxin reductase in adult flies. However, the presence of DNP in the food mixtures prevented SNP-induced changes in thioredoxin reductase and glucose-6-phosphate dehydrogenase activities, as well as uric acid and low-molecular-mass thiol content. The potential mechanisms by which DNP exerts protective effects against SNP toxicity are discussed.

1. Introduction

An imbalance between production and elimination of reactive oxygen species (ROS) in favor of the former and disturbing core and regulatory vital processes is called oxidative stress (Sies, 1991; Lushchak, 2011). Elimination of ROS is provided by antioxidant systems that are usually grouped into two classes: enzymatic defenses such as superoxide dismutase (SOD) and peroxidases including catalase, and nonenzymatic defenses including tocopherols, carotenes, ascorbate, glutathione (GSH), etc. (Halliwell and Gutteridge, 1999; Lushchak, 2011). The mitochondrial electron transport chain is the primary site of endogenous ROS formation (Halliwell and Gutteridge, 1999). It is well known that a shift in respiration that diminishes mitochondrial potential decreases ROS formation. Hence, mild uncoupling of oxidative phosphorylation is one of the mechanisms which could control ROS production by mitochondria (Skulachev, 1998; 2004). In the current study, we used the protonophore, 2,4-dinitrophenol (DNP), at low (nontoxic) concentrations in order to induce mild uncoupling of respiration and the toxicant, sodium nitroprusside (SNP; $Na_2[Fe(CN)_5NO]$), in concentrations known to cause notable effects on fly viability and antioxidant systems to induce oxidative/nitrosative stress in *Drosophila* (Lozinsky et al., 2012b). Sodium nitroprusside is a water-soluble iron nitrosyl complex consisting of a ferrous ion surrounded by five cyanide moieties and a nitrosyl group. In basic research, it has been used mainly to investigate different mechanisms triggered by nitrosative and oxidative stresses (Cardaci et al., 2008; Lozinsky et al., 2012b). In our previous studies on yeast (Lushchak and Lushchak, 2008b) and flies (Lozinsky et al., 2012b), we found that SNP induced mild oxidative/nitrosative stress in both organisms. We also found that the toxicity of SNP might arise not just from its direct effects, but also from its decomposition products such as nitric oxide and iron ions (Lozinsky et al., 2012b).

2,4-Dinitrophenol (DNP) has been long known to be toxic at high concentrations due to its actions in uncoupling oxidative phosphorylation. However, numerous studies have reported that DNP at low concentrations protects neurons against β-amyloid toxicity (De Felice and

Abbreviations: DTNB, 5,5′-dithio-bis (2-nitro) benzoic acid; DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetraacetate; G6PDH, glucose-6-phosphate dehydrogenase; GSH/GSSG, oxidized/reduced glutathione; GST, glutathione-S-transferase; ICDH, NADP-dependent isocitrate dehydrogenase; L-SH, low molecular mass thiols; NADP/NADPH, oxidized/reduced nicotinamide adenine dinucleotide phosphate; PC, protein carbonyls; PMSF, phenylmethylsulfonyl fluoride; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TrxR, thioredoxin reductase.

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Ferreira, 2006), neurodegeneration (Liu et al., 2008; Madeiro da Costa et al., 2010) and other types of insults (Korde et al., 2005). Other studies demonstrated that DNP at nontoxic (low) concentrations stimulated neurite outgrowth, neuronal differentiation and even extended murine lifespan (Caldeira da Silva et al., 2008) as well as lifespan in yeast (Barros et al., 2004) and flies (Padalko, 2005). In addition, Caldeira da Silva et al. (2008) showed that DNP could prevent development of oxidative stress by decreasing ROS levels in mice. Recently, Liu et al. (2008) found that DNP at nontoxic concentrations could stimulate energy metabolism through mechanisms which involve a mild metabolic stress response. Their study also showed that DNP regulated the sirtuin signaling pathway. Finally, similar to calorie restriction, DNP activated eNOS (nitric oxide synthase) and Akt-dependent pathways leading to mitochondrial biogenesis in mice (Cerqueira et al., 2011). Based on the above findings, we decided to investigate the influence of SNP and DNP both separately and together on development and oxidative stress parameters in *Drosophila*

The present study examined the possible protective effects of DNP against SNP-induced toxicity *in vivo* on *D. melanogaster* with the objective of understanding the effects of SNP and DNP on development of the flies and the responses of the antioxidant defense system to SNP and DNP.

2. Materials and methods

2.1. Reagents

Phenylmethylsulphonyl fluoride (PMSF), sodium nitroprusside (SNP), $NADP^+$, reduced glutathione (GSH), 2,4-dinitrophenylhydrazine (DNPH), 2,4-dinitrophenol (DNP), glucose-6-phosphate, isocitrate, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), 5-sulfosalicylic acid (SSA), N,N,N′,N′-tetramethyl ethylenediamine (TEMED), Tris–HCl, 1-chloro-2,4-dinitrobenzene (CDNB) and quercetin were obtained from Sigma–Aldrich Chemie GmbH (Germany). NADPH was from Reanal (Hungary) and guanidine-HCl was from Fluka (Germany). All other chemicals were of analytical grade.

2.2. Drosophila melanogaster stock and media

The *D. melanogaster* strains w^{1118} and white Dahomey (w^{Dah}) were obtained from stock-centers or collaborators. Stock flies and larvae were reared on yeast-corn-molasses (regular) food with constant illumination at 25 ± 1°C. Nipagin 0.2% (methyl-*p*-hydroxybenzoate) was added to the medium to inhibit mold growth. For all time course experiments, control larvae were fed with food containing 10% sucrose, 10% yeast and 1% of agar-agar (SY diet); and experimental groups were fed the same food, but supplemented with SNP (1.0 mM), DNP (0.5 or 1.25 mM) or one of two mixtures (SNP 1.0 mM + DNP 0.5 mM, or SNP 1.0 mM + DNP 1.25 mM).

2.3. Pupation

After egg laying for 6 h, the eggs were transferred to vials containing SY diet either alone (control) or supplemented with DNP, SNP or mixtures of both, as above; about 170 eggs were added per vial containing 20 mL of food. In these vials, eggs hatched and larvae developed until pupation. The total number of eggs and total number of pupated larvae in each vial were counted.

2.4. Effect of test chemicals on larval behavior (pupation height preference)

Changes in larval behavior in response to the experimental chemical exposures were assessed as pupation height preference. Before pupation, larvae climb up the sides of the vials, attach themselves and then pupate. After 4–6 days when all larvae had pupated, pupation heights were measured as the distance from the food surface (pupation on the food surface was scored as zero). The pupation height was measured in millimeters (mm) as described previously (Singh and Pandey, 1991).

2.5. Food intake by larvae

Food intake was measured as described previously (Lozinsky et al., 2012b). Briefly, groups of 10 third-instar larvae were reared on the standard medium and then placed for 24 h on SY diet either without (control) or supplemented with DNP, SNP or mixtures of both, as described above. The diet for this experiment also contained 0.5% FD&C Blue No.1 dye (Brilliant Blue FCF) and the food was poured on Petri dishes. After 24 h of feeding, groups of larvae were homogenized and centrifuged at room temperature at 16,000 g for 15 min. Supernatant samples were removed and absorbance was measured at 629 nm and compared against a calibration curve built with different concentrations of the dye.

2.6. Preparation of extracts of adult flies for metabolic analyses

Two-day-old flies were separated by sex and only females were used for analysis. Flies were homogenized using a Potter–Elvejhem glass homogenizer (1:10 w:v) in cold 50 mM potassium phosphate (KPi) buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM PMSF. After centrifugation at 16,000 g for 15 min at 4 °C in an Eppendorf 5415 R centrifuge (Germany), the supernatants containing water soluble proteins were collected and used for different assays and protein measurements using either a Specoll 211 (Carl Zeiss Jena, Germany), Microlab 300 (Vital Scientific, Netherlands) or SF-46 (LOMO, Russia) spectrophotometer. For uric acid assays supernatants were additionally treated for 15 min at 70 °C in order to inactivate all endogenous enzymes, including uricase.

2.7. Iron determination

The concentration of iron in 2-day old female flies was determined using a colorimetric standard kit with Ferene [3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5′′-disulfonic acid disodium salt] without deproteinization (Cormay, Poland), according to manufacturer's instructions. The iron concentration was expressed as micromoles per gram of fly wet mass.

2.8. Oxidative stress markers

The content of carbonyl groups in water soluble proteins (CP) was measured by determining the amount of 2,4-dinitrophenylhydrazone formed upon the reaction of proteins with DNPH (Lushchak et al., 2009). Protein carbonyl content was calculated from the absorbance maximum of 2,4-dinitrophenylhydrazone measured at 370 nm using an extinction coefficient of 22 mM^{-1} cm^{-1} (Levine et al., 2000). The results are expressed in nanomoles per milligram of protein.

The concentration of uric acid was determined using an enzymatic colorimetric standard kit with uricase and peroxidase (Cormay, Poland), according to manufacturer's instructions. The uric acid concentration was expressed as micromoles per gram of fly wet mass.

Free thiols were measured spectrophotometrically with DTNB at 412 nm as described previously (Lushchak et al., 2011). Total thiol content (the sum of low- and high-molecular-mass thiol-containing compounds) was measured in the resulting supernatants. For measurement of low-molecular-mass thiol-containing compounds (L-SH) content, supernatants were treated with 10% TCA (final concentration), centrifuged for 5 min at 13,000 *g* and the final supernatants were used for the assay. The thiol concentrations were expressed as micromoles of SH-groups per gram of fly wet mass.