



Original Contribution

N-arachidonylglycine causes ROS production and cytochrome c release in liver mitochondria

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ABSTRACT

N-arachidonylglycine (NA-Gly) is an amino acid derivative of arachidonic acid. This compound is structurally related to anandamide (arachidonylethanolamine), which is considered an endogenous ligand of the cannabinoid receptor. NA-Gly is present at relatively high levels in the spinal cord, small intestine, and kidneys and at lower, but remarkable, levels in testes, lungs, and liver. The presence of varying levels in different organs suggests multiple functions in addition to the reported anti-inflammatory and pain suppression actions. Here a study on the interaction of NA-Gly with isolated mitochondria is reported. The results show that micromolar concentrations of NA-Gly cause: (i) an increase in the resting state respiration with both glutamate plus malate and succinate as substrates and (ii) a decrease in either ADP- or uncoupler-activated respiration. Whereas the stimulated resting state respiration was substantially reduced by cyclosporin A (CsA), the NA-Gly-inhibited State 3 respiration was almost unaffected. Measurements by blot analysis showed that NA-Gly caused a CsA-sensitive cytochrome c release. Under these conditions no matrix swelling could be detected. Experiments are also presented showing that NA-Gly caused a respiration-dependent large ROS production, which seems in turn to be responsible for the inhibition of electron transport activity and cytochrome c release.

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Arachidonic acid (AA) is a long-chain polyunsaturated free fatty acid (FFA) whose interaction with mitochondrial membranes has been actively studied. Micromolar concentrations of AA cause: (i) the generation of reactive oxygen species (ROS), associated with the forward electron transport from substrates to oxygen [1–3], and (ii) the release of intermembrane-localized proapoptotic proteins, such as cytochrome c, as a consequence of a Ca^{2+} -dependent increase in mitochondrial membrane permeability [4–6]. These effects are not specific for AA, because other FFAs have similar effects in mitochondria from various tissues [7–12].

Much attention has recently been addressed to the study of the occurrence, roles, and effects of arachidonic acid derivatives. These lipids exert a range of important biological actions and include various inflammatory mediators, such as the prostaglandins and the endocannabinoids, which exhibit analgesic effects. Several studies on the effects of endocannabinoids on mitochondrial functions have been recently reported. The endocannabinoid 2-arachidonyl glycerol has

been shown to induce apoptosis in hepatic stellate cells. Mitochondrial depolarization and increased mitochondrial permeability were demonstrated to result after 2-arachidonyl glycerol treatment of the cells [13]. A study of the effect of N-Arachidonylethanolamine (anandamide), which is considered an endogenous ligand of the brain cannabinoid receptor [14], on some mitochondrial functions has also been reported [15,16]. Anandamide uncouples oxidative phosphorylation and inhibits uncoupled respiration in rat heart mitochondria. A cyclosporin A (CsA)-sensitive anandamide-dependent dissipation of membrane potential and release of accumulated calcium were observed, indicative of the opening of the mitochondrial permeability transition pore (MPTP) [15].

Recently a new class of bioactive molecules has been identified in mammals, namely, the arachidonyl amino acids, in which the carboxylic group of arachidonic acid is bound to the amino group of an amino acid. This family includes at least three members: N-arachidonylglycine (NA-Gly), N-arachidonylalanine, and N-arachidonyl- γ -aminobutyric acid. NA-Gly is widely distributed among mammalian tissues. It is present at relatively high levels in the spinal cord, small intestine, and kidneys (100–140 pmol/g dry wt) and at lower, but remarkable, levels in testes, lungs, and liver (20–30 pmol/g dry wt) [17]. This broad distribution suggests that it may play multiple roles, and interact with a number of target sites, in addition to the reported anti-inflammatory and pain suppression actions [17]. An inhibitory effect of NA-Gly of the glycine transporter GLYT2a subtype,

Abbreviations: AA, arachidonic acid; CCCP, carbonyl cyanide-3 chlorophenylhydrazone; CsA, cyclosporin A; DCFH, dichlorofluorescein; FFA, free fatty acids; FAAH, fatty acid amide hydrolase; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; NA-Gly, N-arachidonylglycine; ROS, reactive oxygen species; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid.

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tentatively related to glycinergic neurotransmission and analgesia, has also been described [18]. Because of these effects, NA-Gly is considered an endocannabinoid-like compound, although it lacks an affinity for cannabinoid receptors [17,19]. It has been proposed that NA-Gly, by inhibiting the fatty acid amide hydrolase (FAAH), the enzyme primarily responsible for the degradation of anandamide, may act as a regulator of the blood levels of this analgesic compound (the “entourage” mode of action) [20,21].

The metabolic origin of NA-Gly is not well understood. Two possible biosynthetic pathways have been proposed: (i) under some conditions NA-Gly may be generated by a pathway involving oxidation of the hydroxyl group of anandamide [22] or (ii) NA-Gly may be synthesized from precursor arachidonic acid and glycine via an enzymatic process involving an arachidonyl-CoA synthase and an enzyme of the acyl-CoA: glycine *N*-acyltransferase family [17]. Very recently, cytochrome *c* was reported to catalyze the *in vitro* synthesis of NA-Gly from arachidonyl-CoA and glycine in the presence of hydrogen peroxide [23].

The described effects of cannabinoids on mitochondrial functions and, in particular, on the mitochondrial route of apoptosis, prompted us to carry out the present investigation aimed at getting some insight into the effects produced by NA-Gly on isolated mitochondria. We report here that NA-Gly causes a substantial increase in resting state respiration and the inhibition of ADP as well as uncoupler-stimulated respiration. A large NA-Gly-dependent ROS production coupled to the forward electron transport from substrates to oxygen was associated with mitochondrial permeability transition (MPT)-dependent cytochrome *c* release.

Materials and methods

Chemicals

Phenylmethanesulfonyl fluoride (PMSF), oligomycin, carbonyl cyanide-3 chlorophenylhydrazone (CCCP), rotenone, antimycin A, safranin-O, alamethicin, arachidonic acid, and 4,4'-diisothiocyanatosilbene-2,2'-disulfonic acid (DIDS) were purchased from Sigma (St. Louis, MO, USA). Cyclosporin A was from Calbiochem (San Diego, CA, USA). NA-Gly was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Mouse anti-cytochrome *c* antibody was purchased from Zymed Laboratories Invitrogen Immunodetection (Carlsbad, CA, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was from Invitrogen-Molecular Probes (Eugene, OR, USA). Horseradish peroxidase (HRP) was from Roche Diagnostics Corp. (Indianapolis, IN, USA).

Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by differential centrifugation using an isolation buffer containing 0.22 M mannitol, 75 mM sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA, and 0.25 mM PMSF [24]. The final mitochondrial pellet was resuspended in the same isolation buffer at a protein concentration of 50–60 mg/ml as determined by the biuret method, using bovine serum albumin as standard. Mitochondrial particles were prepared by freezing and thawing the mitochondria three times.

Measurement of oxygen consumption rate

The respiratory activity of liver mitochondria was measured polarographically with a Clark-type electrode, in an all-glass reaction chamber stirred magnetically, at 25°C. Mitochondrial proteins were suspended at a final concentration of 0.5 mg/ml in a medium containing 75 mM sucrose, 50 mM KCl, 30 mM Tris-Cl (pH 7.4), 2 mM KH_2PO_4 , 2 mM MgCl_2 , and 10 μM EGTA (Buffer A). Resting state respiration was started on the addition of glutamate plus malate (5 mM each) or succinate (10 mM) in the presence of 2 $\mu\text{g}/\text{ml}$ rotenone. State 3 and uncoupled respiration were obtained by adding

0.5 mM ADP and 0.5 μM CCCP, respectively. Other additions are specified in the figure legends.

Measurement of redox activities in mitochondrial particles

NADH-cytochrome *c* oxidoreductase (complex I + III) and succinate-cytochrome *c* oxidoreductase (complex II + III) activity was determined spectrophotometrically by following the reduction of cytochrome *c* at 550–540 nm. NADH and succinate oxidase activity was measured polarographically [25].

Measurement of membrane potential

The membrane potential ($\Delta\Psi$) in intact mitochondria was measured as previously described [25] after the safranin-O fluorescence quenching at 525 nm (excitation) and 575 nm (emission) with a Jasco FP 6200 spectrofluorimeter. Rat liver mitochondria (0.25 mg/ml) were suspended in 1 ml of Buffer A without MgCl_2 . Swelling was initiated by the addition of 1 $\mu\text{g}/\text{ml}$ rotenone, at 25°C. The membrane potential was generated by the addition of 10 mM succinate.

Mitochondrial swelling

Changes in absorbance of mitochondria were monitored at 540 nm in an Agilent 8453 diode-array spectrophotometer. Mitochondria were suspended at 0.25 mg/ml in Buffer A without MgCl_2 . Swelling was initiated by the addition of Ca^{2+} or NA-Gly to the mitochondria suspension supplemented with 10 mM succinate (plus 1 $\mu\text{g}/\text{ml}$ rotenone).

Detection of mitochondrial H_2O_2 production

The rate of mitochondrial hydrogen peroxide production was estimated by measuring the linear fluorescence increase (excitation at 475 nm, emission at 525 nm) induced by the H_2O_2 oxidation of dichlorofluorescein (DCFH) to the fluorescent compound dichlorofluorescein in the presence of HRP. DCFH was obtained from its commercially available diacetate ester (DCFH-DA) by alkaline hydrolysis immediately before the experiment [26]. Mitochondrial proteins (0.25 mg) were suspended in 1 ml of Buffer A also containing 1.36 μM DCFH and glutamate plus malate (5 mM each) or succinate (10 mM) as respiratory substrate. The reaction was started by the addition of 0.4 μM HRP and the fluorescence signal was measured. Conversion of fluorescence units to nanomoles of H_2O_2 produced was performed by measuring the fluorescence changes upon addition of known amounts of H_2O_2 .

Detection of cytochrome *c* release

Freshly isolated mitochondria (0.5 mg/ml) were suspended in Buffer A supplemented with 2 $\mu\text{g}/\text{ml}$ rotenone and incubated for 2 min at 25°C. NA-Gly, succinate (10 mM), and ADP (0.5 mM) were subsequently added. Six minutes after succinate addition the mitochondria were spun down at 4000 g for 10 min at 4°C and the resulting supernatants were centrifuged at 100,000 g for 15 min. The supernatants of the second centrifugation were concentrated using Millipore Ultrafree-4 centrifugal filters and used for the detection of cytochrome *c* by SDS-PAGE as described [27]. The gel was blotted onto a nitrocellulose membrane and probed by a mouse monoclonal anti-cytochrome *c* antibody. Immunoblot was performed with HRP-conjugated anti-mouse antibody using the chemiluminescence ECL kit. Relative optical densities of bands were quantified by densitometric analysis.

Lipid extraction and analysis

Mitochondria (10 mg protein) were suspended in Buffer A supplemented with rotenone (2 $\mu\text{g}/\text{ml}$), succinate (10 mM), and

ADP (0.5 mM) and incubated for 0, 3, or 6 min with NA-Gly used at 30 nmol/mg protein. Total lipids were extracted using the Bligh and Dyer method [28]. The extracts were carefully dried under N_2 before being weighed and then dissolved in chloroform. Total lipid extracts were analyzed by TLC on silica gel 60A plates (Merck, 20 × 10 cm, layer thickness 0.2 mm). Lipids were eluted with chloroform/methanol/acetic acid/water 85/15/10/3.5 (v/v) (solvent for polar lipids) or hexane/diethyl ether/acetic acid 35/15/0.5 (v/v) (solvent for neutral lipids) and detected by spraying with 5% sulfuric acid followed by charring at 120°C. The quantitative analyses of lipid contents were performed by video densitometry, using the software ImageJ (<http://rsb.info.nih.gov/ij/>).

Results

Mitochondrial respiration and membrane potential

The effects of NA-Gly on oxygen consumption activity of rat liver mitochondria were examined. A dual effect was observed: stimulation of substrate supported (resting state) respiration and inhibition of either ADP or uncoupler stimulated respiration (Fig. 1A). The concentration-dependent stimulation of resting state respiration was observed with either succinate (plus rotenone) or glutamate plus malate as substrate, with the effect being completely reversed by the addition of BSA (Fig. 1B). NA-Gly increased the oxygen consumption

rate as well, if added after substrate pulse. Separate experiments showed that the NA-Gly-dependent stimulation of succinate-supported resting state respiration was unaffected by either atractyloside or glutamate or even by a combination of these two compounds. This would suggest that the ADP/ATP antiporter as well as the aspartate/glutamate antiporter, which are thought to mediate the uncoupling effect of free fatty acids [7,29,30], are apparently not involved in the NA-Gly effect. Similarly, GDP used at concentrations up to 0.5 mM did not decrease the NA-Gly-induced stimulation of the resting state respiration. This finding rules out a possible involvement of uncoupling proteins in the observed effect (not shown).

The inhibition of activated respiration seemed to be concentration dependent as well, irrespective of whether ADP or CCCP (in the presence of oligomycin) was used to activate the respiration (Fig. 1C). As a result of these effects NA-Gly caused a marked drop in the respiratory control ratio measured as ADP-stimulated vs succinate- or glutamate plus malate-supported respiration. The ratio calculated as uncoupler-stimulated vs oligomycin-inhibited respiration was similarly lowered (Fig. 1D). Experiments in which the initial rate of respiration was measured by adding succinate to ADP-supplemented mitochondria immediately after the addition of NA-Gly produced results similar to those presented in Figs. 1A and 1C.

The data reported in Fig. 2 highlight a striking difference between the effects produced on oxygen consumption by NA-Gly and arachidonic acid. It is shown that CsA greatly decreased (from around

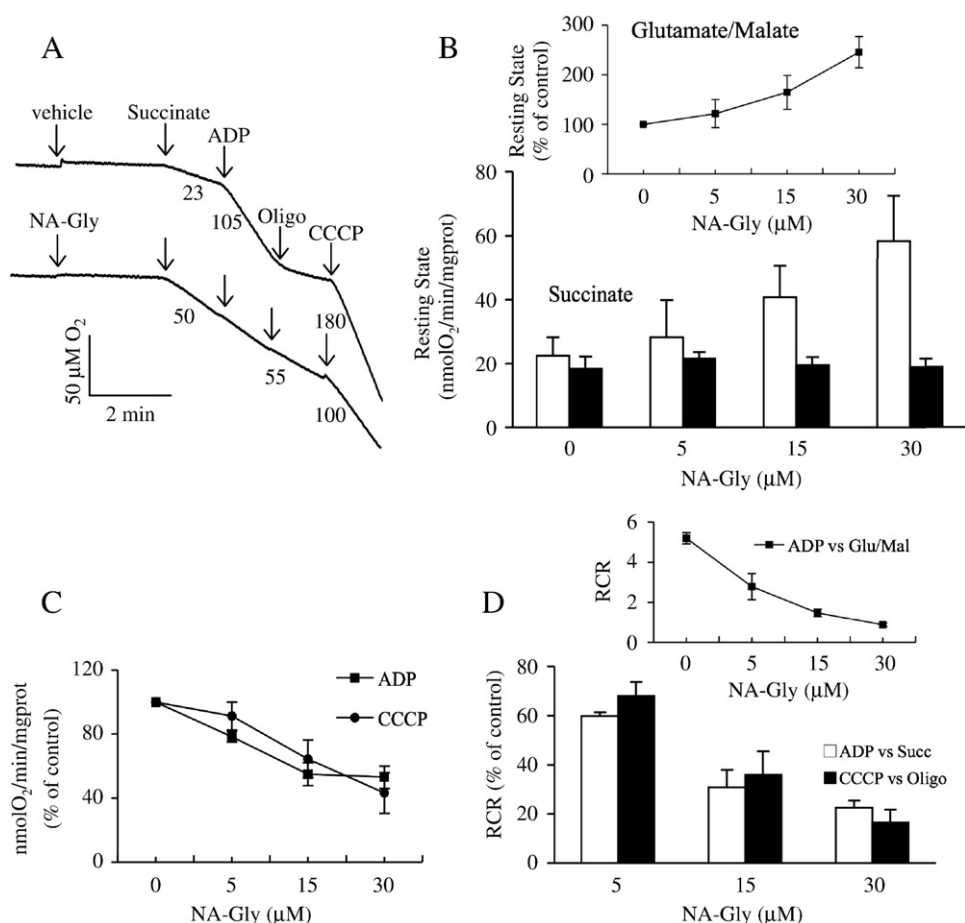


Fig. 1. Effects of NA-Gly on oxygen consumption in rat liver mitochondria. Respiration was initiated by the addition of succinate (plus rotenone) or glutamate plus malate to mitochondria preincubated with the indicated concentrations of NA-Gly. (A) Traces of oxygen consumption in succinate-supplemented mitochondria. NA-Gly (vehicle in the control) was used at 15 μM. Where indicated ADP (0.5 mM), oligomycin (2 μg/ml), and CCCP (0.5 μM) were added. Numbers on the traces refer to the rate of oxygen consumption as nmol O₂ · min⁻¹ · mg protein⁻¹. (B) Resting state respiration in succinate- or glutamate plus malate (inset)-oxidizing mitochondria. BSA (0.1%, w/v) (black bars) was added 2 min after succinate. (C) Effect of NA-Gly on ADP- or CCCP-stimulated respiration rate. (D) Respiratory control ratio (RCR), measured at the indicated NA-Gly concentrations, as ADP-stimulated vs succinate- or glutamate plus malate (inset)-supported respiration. RCR is also reported as CCCP-stimulated vs oligomycin-inhibited respiration, with succinate as substrate. Values reported are means ± SD from 5 to 10 separate experiments.

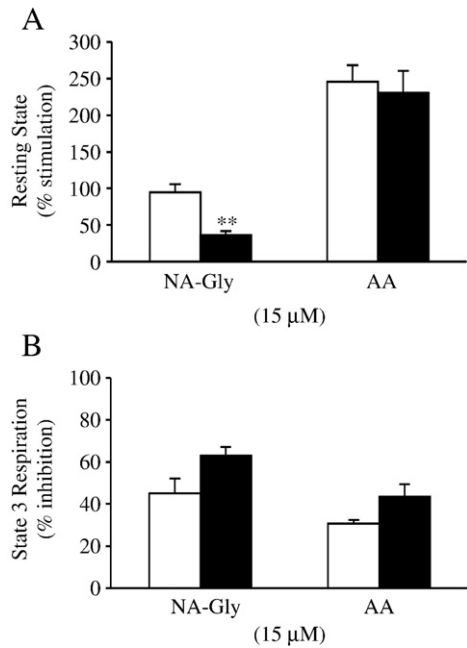


Fig. 2. Effects of CsA on the respiratory activity in NA-Gly- and arachidonic acid-treated mitochondria. The experimental conditions were those described in the legend to Fig. 1A. Concentrations used were 15 μM NA-Gly, 15 μM AA, and 2 μM cyclosporin A (black bars). Mean values ± SEM from five to seven separate experiments are reported (***p* < 0.001).

90 to 30%) the NA-Gly-dependent stimulation of resting state respiration (Fig. 2A). This effect is suggestive of the activation of MPT by NA-Gly. CsA, in contrast, did not change substantially the inhibition exerted by NA-Gly on the rate of oxygen consumption under State 3

respiration. A further small inhibitory effect could even be observed (Fig. 2B). Arachidonic acid, used at the same concentration, caused a stronger stimulation of the resting state respiration, indicative of its high protonophoric property (Fig. 2A). Neither the resting state nor the State 3 respiration measured in the presence of arachidonic acid was affected significantly by CsA.

Measurements of redox activities in mitochondrial particles revealed that NA-Gly, used in the same concentration range as described for Fig. 1, did not cause any appreciable inhibition of the activity of complexes I + III and II + III. NADH and succinate oxidase activities were similarly unaffected (not shown). It is noteworthy that complex I, complex I + III, and NADH oxidase activities in mitochondrial particles have been reported to be inhibited by free arachidonic acid and its derivative anandamide [1,16].

Fig. 3 shows traces of safranin fluorescence quenching ensuing upon addition of succinate to mitochondria supplemented with NA-Gly or arachidonic acid. In the presence of 7.5 μM NA-Gly (30 nmol/mg protein), the membrane potential trace was very stable and started declining slowly. As the concentration was increased to 15 μM, the membrane potential dropped some minutes after succinate addition (Fig. 3, trace b). MPT activation seemed to contribute to this effect as indicated by the finding that CsA delayed markedly the drop in the potential (trace c). Arachidonic acid was much more effective in membrane potential dissipation, which was neither prevented nor delayed by CsA (traces d and e).

Cytochrome *c* release and matrix swelling

The effects of NA-Gly on cytochrome *c* release from mitochondria were measured (Fig. 4). NA-Gly at 15 μM caused a CsA-sensitive cytochrome *c* release amounting to around 85% of the maximal release induced by alamethicin, a channel-forming antibiotic that causes maximal release of intermembrane proteins [31]. The addition of DIDS, reported to be a voltage-dependent anion channel (VDAC)

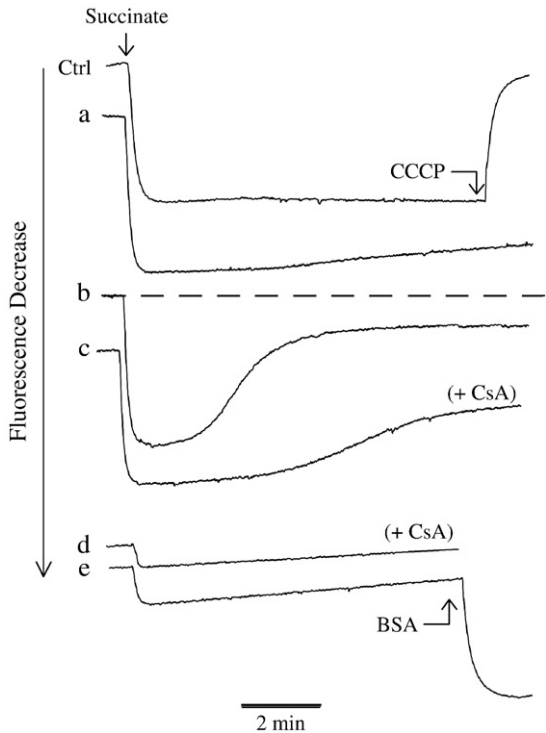


Fig. 3. Effects of NA-Gly on mitochondrial transmembrane potential. ΔΨ was generated by succinate addition to mitochondria supplemented with NA-Gly or arachidonic acid. Concentrations used were 7.5 μM NA-Gly (trace a), 15 μM NA-Gly (traces b and c), and 15 μM arachidonic acid (traces d and e), and vehicle was used in the control (Ctrl). Where indicated 2 μM CsA was present. Dashed line: as in b without mitochondria. BSA and CCCP were used at concentrations of 0.1% (w/v) and 0.25 μM, respectively.

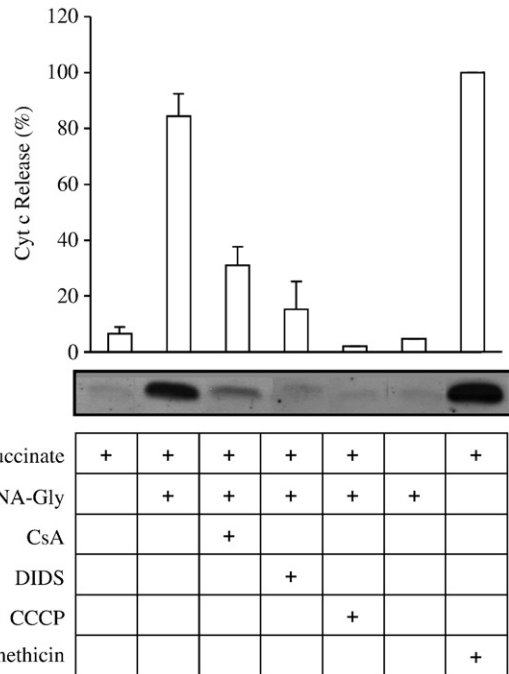


Fig. 4. NA-Gly caused cytochrome *c* release in energized mitochondria. For experimental conditions and details see Materials and methods. Values from densitometric analysis of the bands are expressed as the percentage of the maximal release induced in the presence of alamethicin (75 μg/mg protein). NA-Gly was used at 15 μM. Where indicated CsA (2 μM), DIDS (50 μM), and CCCP (0.5 μM) were present. Values reported are means ± SD from four to seven separate experiments.

blocker, inhibited the release process as well. Interestingly, cytochrome c was not released in the absence of respiratory substrate or in the presence of the uncoupler CCCP.

Absorbance measurement at 540 nm showed that NA-Gly addition caused a short-lasting CsA-insensitive light scattering decrease in the mitochondrial suspension (Fig. 5A). Furthermore this absorption change was respiration independent, as it occurred even before succinate addition (Fig. 5B). Mitochondria did, however, undergo CsA-sensitive matrix swelling when pulsed with 30 μM Ca^{2+} , this representing our internal positive control.

Reactive oxygen species generation

The effect of NA-Gly on ROS generation associated with the forward electron transport was analyzed in mitochondria oxidizing succinate (plus rotenone) or glutamate plus malate. Fig. 6A shows that hydrogen peroxide generation increased with increasing concentrations of NA-Gly. The results summarized in Table 1 show that antimycin enhanced basal ROS generation associated with succinate (plus rotenone) or glutamate plus malate oxidation. Importantly, ROS production consequent to complete inhibition of the respiratory chain activity by antimycin was further enhanced by NA-Gly. As expected,

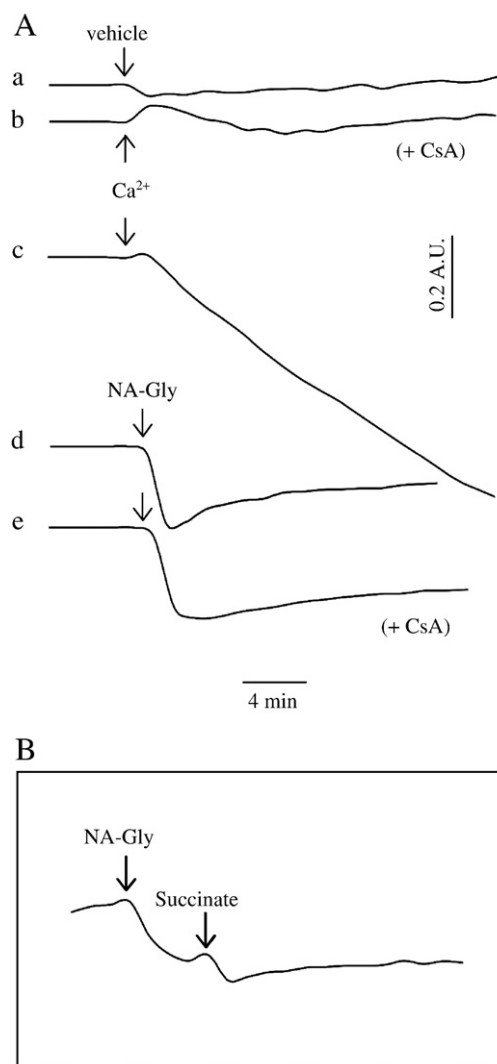


Fig. 5. Effects of NA-Gly on mitochondrial swelling. (A) Swelling in succinate-energized mitochondria was started by the addition of 30 μM Ca^{2+} (traces b, c) or 15 μM NA-Gly (traces d, e). Vehicle was used in the control (trace a). Traces b and e were obtained in the presence of 2 μM CsA. (B) Absorbance trace obtained by the addition of 15 μM NA-Gly followed by 10 mM succinate.

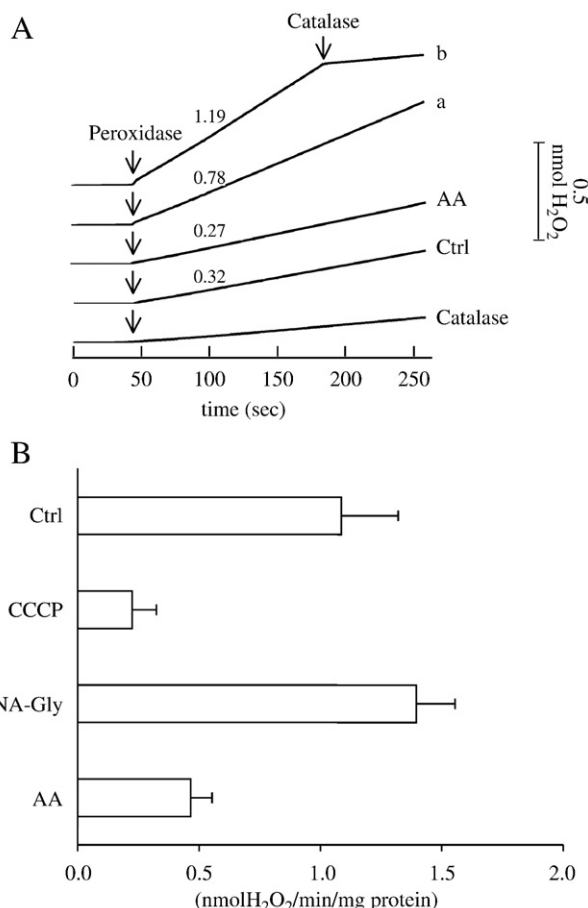


Fig. 6. Effects of NA-Gly on H_2O_2 production in respiring mitochondria. (A) H_2O_2 production associated with the forward electron transport from succinate to oxygen. Mitochondrial suspension (0.25 mg protein/ml) was supplemented with succinate (plus rotenone) and NA-Gly at 7.5 (trace a) and 15 μM (trace b) (vehicle in the control). Arachidonic acid was used at 15 μM (trace AA). The reaction was started by the addition of horseradish peroxidase. Where indicated catalase (1.6 μM) was added. Numbers on the traces refer to the rate of H_2O_2 production as $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. (B) H_2O_2 production associated with the reverse electron transport from succinate to complex I. Conditions were as in (A) except that rotenone was omitted from the reaction mixture. Where indicated 0.25 μM CCCP, 15 μM NA-Gly, and 15 μM AA were present in the mitochondrial suspension. Values reported are initial rate \pm SD (from four separate mitochondrial preparations) of H_2O_2 production as $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. For other experimental conditions see Materials and methods.

arachidonic acid, at the concentration used (see [1,3]), did not cause any ROS production increase (Fig. 6A).

The addition of succinate (in the absence of rotenone) to mitochondria under nonphosphorylating conditions feeds electrons to complex I and causes a large $\Delta\Psi$ -dependent ROS production associated with the so-called reverse electron transport [32,33]. It was in fact uncoupler sensitive and greatly decreased by arachidonic acid, the uncoupling properties of which are well established (Fig. 6B) [1–3].

Table 1

Hydrogen peroxide generation associated with forward electron transfer in mitochondria oxidizing succinate (plus rotenone) or glutamate plus malate: effects of antimycin A and NA-Gly

	H_2O_2 production ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$)	
	Succinate	Glutamate + malate
Control	0.33 ± 0.05	0.22 ± 0.05
+ Antimycin A	0.86 ± 0.05	1.62 ± 0.05
+ Antimycin A + NA-Gly 7.5 μM	2.00 ± 0.27	2.55 ± 0.40
+ Antimycin A + NA-Gly 15 μM	2.55 ± 0.27	2.92 ± 0.25

Experimental conditions as for Fig. 6A. Where indicated antimycin A (1 $\mu\text{g}/\text{ml}$) and NA-Gly (7.5 and 15 μM) were present.

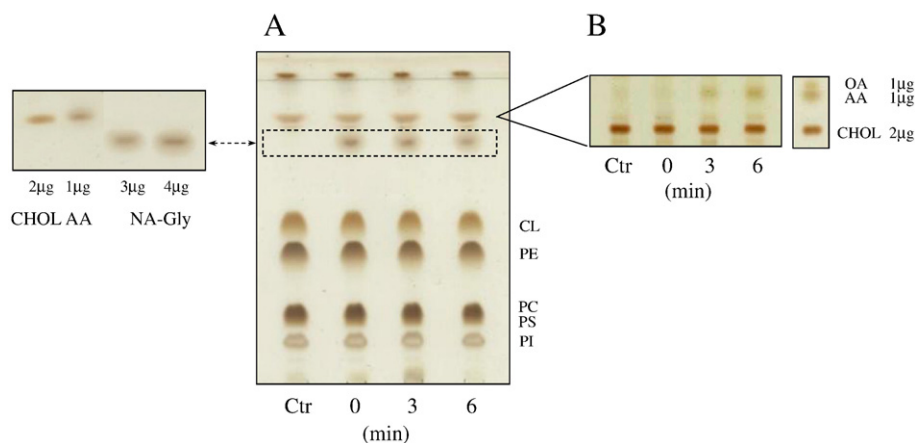


Fig. 7. Lipid analysis of NA-Gly-treated mitochondria. Lipids were extracted from mitochondria incubated for 0, 3, and 6 min with NA-Gly (vehicle in the control). Thin-layer chromatography was performed with two elution systems: (A) an acidic solvent system and (B) a solvent for neutral lipids. 150 μ g of total lipid extract was loaded in each lane. Abbreviations for polar lipids: CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol. Dashed box highlights NA-Gly hydrolysis. Standards of cholesterol (CHOL), arachidonic acid (AA), oleic acid (OA), and NA-Gly are shown. For other conditions and details see [Materials and methods](#).

Under these conditions NA-Gly caused instead a small increase in the initial rate of hydrogen peroxide production.

Lipid analysis

In [Fig. 7](#) is shown an experiment aimed at evaluating whether and to what extent NA-Gly underwent metabolism in liver mitochondria under our conditions. TLC analysis of lipid extract from mitochondrial suspension showed that added NA-Gly migrated in the region of neutral lipids. Densitometry determination of the blots revealed that the NA-Gly concentration decreased by less than 10 and 20% after 3 and 6 min incubation with respiring mitochondria, respectively ([Fig. 7](#), dashed box). In contrast, the intensity of the cholesterol band increased slightly as a result of its overlapping with that of arachidonic acid produced by NA-Gly hydrolysis ([Fig. 7A](#)). This conclusion is substantiated by the results of the experiment shown in [Fig. 7B](#), in which, by modifying the elution solvent, the cholesterol band could be split, thus evidencing an increase in the arachidonic acid band intensity in the lipid extract of mitochondria incubated with NA-Gly. Such increase matched closely the observed NA-Gly decrease. No other free fatty acids were apparently produced under these conditions, neither did the addition of NA-Gly to respiring mitochondria cause changes in the general profile of polar lipids.

Discussion

While studying the effects on mitochondrial functions of a compound such as NA-Gly, it is worth considering the possible effects caused by products of its metabolism. The liver mitochondrial FAAH catalyzes the hydrolysis of NA-Gly [20,34], thus releasing free arachidonic acid, whose effects on mitochondria have been extensively studied [1–7,10] and which was used here as a comparison. The *in vivo* steady-state effective NA-Gly concentration is thus dependent on its synthesis and hydrolysis rates. With regard to this, it has to be considered that NA-amino acid as well as *N*-acylethanolamine derivatives, anandamide among others, are both substrates for FAAH and compete against each other. Based on the K_m of NA-Gly at COX-2 and on the concentrations to inhibit FAAH, Wiles et al. [18] have estimated that the concentration of NA-Gly at other potential sites will vary between 5 and 24 μ M. Furthermore, it has to be considered that local concentrations of lipid molecules, such as NA-Gly, may be higher than those observed.

In this work we have examined the effects produced by these concentrations of NA-Gly on liver mitochondrial functions. Although we have shown that NA-Gly underwent partial hydrolysis ([Fig. 7](#)) with

the release of arachidonic acid, the data presented (except those relative to the measurement of cytochrome *c* release, see below) are from experiments in which the initial rates of the individual reactions were measured after a short incubation of NA-Gly with mitochondria. Furthermore, by comparing the effects elicited by FFA, arachidonic acid in particular, and NA-Gly on mitochondrial functions, we can argue that they differ substantially in many aspects. Thus, the effects described here can be considered specific and genuine for NA-Gly.

Arachidonic acid, like other free fatty acids, increased the resting state oxygen consumption because of its protonophoric property ([Fig. 2](#)) [1–3,7,10]. Consistently arachidonic acid strongly decreased the membrane potential-supported ROS production associated with the reverse electron transport from succinate to complex I ([Fig. 6B](#)) [3]. NA-Gly in contrast did not decrease ROS production under reversed electron transport conditions, and neither, with respect to arachidonic acid, did it exhibit comparable protonophoric properties ([Figs. 2, 3, and 6](#)).

The increase in the resting state respiration by NA-Gly, but not by arachidonic acid, was largely sensitive to CsA, this indicating that MPTP opening may be induced by NA-Gly. Membrane potential determination in the presence of an NA-Gly concentration causing a CsA-sensitive stimulation of the resting state respiration revealed that $\Delta\Psi$ was stable for several minutes. This observation may be explained by considering that pore opening may be transient, leading to a transient $\Delta\Psi$ loss and inner membrane permeabilization (flickering pores). The lack of matrix swelling further suggests that NA-Gly induced MPTP opening in a lower conductance mode. However, under these conditions, the process brought about membrane permeabilization, which caused in turn CsA-sensitive cytochrome *c* release ([Fig. 4](#)).

It is well known that MPTP opening may be induced by Ca^{2+} overload or oxidative stress [35]. Again, at variance with the effect exerted by free arachidonic acid [2,4,6], NA-Gly-dependent cytochrome *c* release did not require Ca^{2+} overload. Incubation mixtures used here were Ca^{2+} free, and EDTA was also used throughout the mitochondrial preparation. Thus, it can be argued that the large ROS production we measured on interaction of NA-Gly with mitochondria is mainly responsible for membrane permeabilization and cytochrome *c* release. The finding that the process was inhibited by DIDS points out an involvement of VDAC, another PTP complex protein of the outer membrane, in the release process. Our data support the notion of a specific targeting of ROS to VDAC. This has indeed been demonstrated by using VDAC antibodies that specifically block VDAC channel activities, thereby blocking ROS-induced MPTP opening and cytochrome *c* release [36,37]. ROS-dependent cardiolipin oxidation has been considered the initial step in the release process, causing

detachment of cytochrome *c* from the outer surface of the inner membrane [38].

Similar to the effects of *N*-acetyl sphingosine (*C*₂-ceramide) on heart mitochondria [39], NA-Gly-dependent cytochrome *c* release required not only respiring mitochondria, but also an established membrane potential (Fig. 4). This finding, while excluding that the release process might result from a detergent effect of NA-Gly on the outer membrane, may provide an explanation for the lack of effect of NA-Gly on the redox activities measured in particles prepared by freezing and thawing of mitochondria. Based on the above observations, it seems that NA-Gly is active only in intact respiring mitochondria, where, we are tempted to speculate, the membrane potential would drive NA-Gly to the effective interaction within the membrane.

ROS production is likely to be caused by the NA-Gly-dependent inhibition of the electron transport activity in intact mitochondria (Fig. 1). However, the finding that NA-Gly caused ROS production at a rate even higher than that observed in the presence of antimycin, i.e., with the respiration chain completely inhibited (Table 1), indicates that the inhibition of the respiratory chain is not the only factor stimulating ROS generation under these conditions. Other authors have indeed reported similar observations [3]. The current hypothesis is that conditions leading to increased membrane fluidity may facilitate the accessibility of oxygen to electron-donating sites of the respiratory chain and, consequently, the release of superoxide anion [3]. This effect has actually been considered for classical and nonclassical cannabinoids, when they were shown to affect the activity of many membrane-associated enzymes through effects not related to an action at the cannabinoid receptor [18,40,41]. Consistently, the small CsA-insensitive absorbance decrease at 540 nm we observed on addition of NA-Gly to mitochondria (Fig. 5), as also found for short-chain ceramide [39] and anandamide [15], may represent the consequence of the membrane fluidizing effect exerted by this compound. Of course, the effect of NA-Gly on lipid ordering and membrane fluidity remains to be directly ascertained.

As shown here NA-Gly inhibited both ADP-stimulated and CCCP-uncoupled respiration (Fig. 1). ROS, rather than cytochrome *c* loss, are probably responsible for this inhibition. In fact, CsA, while preventing cytochrome *c* release and largely reducing the increase in the resting state respiration caused by NA-Gly, did not prevent the inhibition of oxygen consumption in the activated state (Fig. 2). Relevant to this point are the reports showing an inhibitory effect attributable to ROS generation on the activity of the respiratory complex enzymes [42].

NA-Gly has been recently reported to represent an endogenous ligand of the G-protein-coupled receptor GPR92, thus possibly mediating intercellular signaling directly in the sensory nervous system [43]. However, the principal mechanism for NA-Gly analgesic action seems to be mediated through reducing anandamide degradation and enhancing cannabinergic signaling [20,21].

In addition to anti-inflammatory and pain control actions, anandamide and other *N*-acylethanolamines have been reported to promote apoptosis and inhibit cell proliferation [44–46]. There is evidence for a causal relationship between ROS production and cell death induced by anandamide. Sarker et al. have in fact shown that anandamide induced *N*-acetyl-L-cysteine preventable apoptosis of PC-12 cells by increasing the superoxide level [47]. Furthermore, cerebral lipoperoxidation under ex vivo conditions was observed after anandamide administration in rats [48]. ROS-mediated anandamide-induced hepatic stellate cell death was subsequently showed by Siegmund et al. [49].

In this study we found a large ROS production increase after treatment of mitochondria with NA-Gly. A Ca²⁺-independent, CsA- and DIDS-sensitive, MPT mechanism was found to be activated, which caused in turn cytochrome *c* release. Once in the cytosol cytochrome *c* is well recognized to drive the biochemical execution of apoptosis

through the activation of the caspase system. Thus NA-Gly appears as a lipid signaling molecule promoting cell death.

Our data suggest that mitochondrial-generated ROS represent the mediators of this NA-Gly function and may also be involved in the anandamide and cannabinoid action in general. Mitochondrial-produced ROS have in fact been found to induce apoptosis in hepatic cells [13].

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