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Different effects of SNP and GSNO on mitochondrial $O_2^{\cdot -}/H_2O_2$ production

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Abstract Sodium Nitroprusside (SNP) and S-Nitrosoglutathione (GSNO) differently affect mitochondrial H_2O_2 release at Complex-I. mM SNP increases while GSNO decreases the release induced by succinate alone or added on top of NAD-linked substrates. Stimulation likely depends on Nitric Oxide ($^{\cdot}NO$) (released by SNP but not by GSNO) inhibiting cytochrome oxidase and mitochondrial respiration. Preincubations with SNP or high GSNO (10 mM plus DTE to increases its $^{\cdot}NO$ release) induces an inhibition of the succinate dependent H_2O_2 production consistent with a $^{\cdot}NO$ dependent covalent modification. However maximal inhibition of the succinate dependent H_2O_2 release is obtained in the presence of low GSNO (20–100 μM), but not with SNP. This inhibition appears independent of $^{\cdot}NO$ release since μM GSNO does not affect mitochondrial respiration, or the H_2O_2 detection systems and its effect is very rapid. Inhibition may be partly due to an increased removal of $O_2^{\cdot -}$ since GSNO chemically competes with NBT and cytochrome C in $O_2^{\cdot -}$ detection.

Keywords Mitochondrial H_2O_2 release · Complex-I (Cplx-I) · Succinate · Nitric oxide · S-nitrosoglutathione · Sodium nitroprusside · Superoxide

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Introduction

Nitric oxide ($^{\cdot}NO$) plays an important role in the regulation of vascular function, activating cyclic guanosine monophosphate (cGMP)-dependent signaling pathways. Excess $^{\cdot}NO$ production may induce nitrosative stress that is also somehow linked to oxidative stress. Oxidative stress in vivo can result from a reduction in endogenous antioxidant processes, excess reactive oxygen species (ROS) generation, or imbalances between antioxidants and pro-oxidant events (Sun et al. 2006).

Besides producing cGMP, $^{\cdot}NO$ can directly modify sulfhydryl residues as low MW thiols like GSH, producing GSNO or protein thiols emerging as an important post-translational protein modification. $^{\cdot}NO$ interaction with haeme proteins as cytochrome oxidase of mitochondria leads to inhibition of the respiratory chain. These actions could result in different and also opposite effects on ROS production.

Reactive nitrogen species (RNS) and ROS may interact as in peroxynitrite generation from the reaction of $^{\cdot}NO$ and superoxide, peroxynitrite being a very strong cytotoxic oxidant, which can irreversibly damage cells by oxidation of free thiols, nitration of tyrosine residues, and lipid peroxidation. In cardiac myocytes, ROS and RNS induce stress-signaling pathways involved in mitochondrial dysfunction, and intracellular Ca^{2+} overload.

Mitochondrial Complex-I (Cplx-I) is recognized as one of the main sources of superoxide/H_2O_2 the features of which have been extensively studied in different kinds of mitochondria. Cplx-I appears either positively or negatively

modulated by several factors so the rate of H_2O_2 generation is highly variable. It is low with NAD-linked substrates; succinate determines a high rate of rotenone sensitive H_2O_2 production, decreased by membrane depolarization (Muller et al. 2008; Starkov 2008; Murphy 2009; Zoccarato et al. 2007). Submillimolar succinate concentrations increase the generation of H_2O_2, unmodified by the contemporary oxidation of NAD-linked substrates (glutamate/malate) (Muller et al. 2008; Zoccarato et al. 2007; Zoccarato et al. 2005). We reported recently that the succinate generated H_2O_2 is inhibited by malate (Muller et al. 2008; Zoccarato et al. 2009), palmitoyl-CoA (Bortolami et al. 2008) and by some propargylamine containing compounds (Zoccarato et al. 2008) and activated by low cyanide concentrations (Zoccarato et al. 2009). The leak of electrons to oxygen is maximal when ETC is more highly reduced. This can be accomplished by several experimental conditions both unphysiological (use of inhibitors of the ETC like antimycin A and CN^-), or physiopathological, as depletion of cytochrome C like in ischemia and apoptosis (Chen and Lesnefsky 2006; Kushnareva et al. 2002) or in the presence of inhibitors of cytochrome oxidase. The small molecules CN^-, and also CO and ·NO produced in biological metabolism share the inhibitory action on mitochondrial cytochrome oxidase (D'Amico et al. 2006), thus their presence might enhance the leak of electrons from Cplx-I resulting in enhanced ROS formation.

The effects of SNP and GSNO, both ·NO releasing agents, although with different kinetic characteristics and efficacy, were studied on rat heart and brain mitochondrial H_2O_2 production to determine how ·NO and ROS may interplay in inducing oxidative stress. GSNO is a physiological metabolite of GSH and ·NO (Khan et al. 2009; Gow et al. 1997; Schrammel et al. 2003) whose known effects are also to prevent ischemic damage (Lima et al. 2009; Chiueh 1999) and brain trauma (Khan et al. 2009).

Unlike other classes of ·NO donors, GSNO is a stable compound and does not decompose spontaneously; it requires additional agents or enzymes, including GSNO reductase or the thioredoxin system (Zeng et al. 2001; Sarkela et al. 2001; Steffen et al. 2001), for its metabolism. Concerning mitochondrial H_2O_2 release the action of ·NO on cytochrome oxidase is expected to increase the production of superoxide/H_2O_2 at Cplx-I, similarly to CN^-. SNP effectively increased H_2O_2 release but GSNO acted oppositely. The study tries to discriminate between the different actions of SNP and GSNO to find the more probable mechanism underlying similar but sometimes opposite effects. We will also show that at low physiological concentration the main effect of GSNO is a direct inhibition of H_2O_2 release, possibly exerted upon interaction with Cplx-I or as a superoxide removing agent. We will also show that SNP (and high concentrations of GSNO in the presence of DTE) may act as negative modulators of H_2O_2 release when preincubated with mitochondria and washed out, probably both inducing a similar protein covalent modification.

Materials and methods

Reagents Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was from Molecular Probes, Horseradish peroxidase (HRP) (grade I;EC1.11.1.7) glucose oxidase from Aspergillus Niger (E.C. 1.1.3.4), and xanthine oxidase from butter milk (EC1.2.3.2) thymol free catalase (C-40), a highly active purified preparation from CuZn superoxide dismutase of Sigma was a generous gift of Dr L.Di Paolo, fatty acid free Bovine Serum Albumin (BSA), xanthine, S-nitrosoglutathione (GSNO) and Sodium Nitroprusside (SNP) from Sigma-Aldrich. All other reagents were of analytical grade.

Preparation of mitochondria

Rat heart mitochondria were isolated from 6 to 7 weeks old rats with the Polytron homogenizer procedure essentially as described (Palmer et al. 1977). BSA 0.5 mg/ml was included in all isolation media. Final mitochondrial pellet was suspended in 0.25 M mannitol, 0.2 mM EGTA and 10 mM Hepes pH 7.3. After protein determination BSA (10 mg/ml) was added to the concentrated suspension (30–40 mg/ml determined by the Gornall procedure).

Brain mitochondria were isolated from cerebral cortices of 6–7 weeks old rats as described (Zoccarato et al. 2004).

Standard incubation Unless otherwise indicated mitochondria (0.3 mg/ml) were incubated at 30 °C in the standard incubation medium: 125 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 100 μM EGTA, 20 mM HEPES, pH 7.2 containing 1 mg/ml defatted bovine serum albumin (BSA). Mitochondrial preincubation without (untreated) or with 2 mM SNP or with 10 mM GSNO in the presence of 100 μM DTE were performed for 30 min in 10 mg/ml mitochondrial suspension at room temperature in the same incubation medium. Reactions were stopped by 10× dilution in ice cold medium and excess reagents was removed after centrifugation (10 min 10,000×*g*). Treated and untreated mitochondrial pellets resuspended in the incubation medium were used as above indicated for the subsequent determination.

Nitrite detection NO_2^- was detected in the supernatants (5 min 18,000×*g*) of the mitochondrial suspension at the end of the incubation with the Griess method (Miranda et al. 2001).

Measurement of mitochondrial respiration Oxygen consumption was monitored with a Clark-type oxygen elec-

trode in a 1.6 ml closed chamber with continuous stirring with 0.3 mg/ml in the standard incubation buffer with the indicated additions.

Hydrogen peroxide measurement Hydrogen peroxide was measured essentially as in (Zoccarato et al. 2005; Mohanty et al. 1997) with 40 μM Amplex Red and 6 μg/ml HRP (1 unit) on a Fluoroskan Ascent FL plate reader in 24 wells plates with the indicated concentration of substrates. The reaction was started with addition of the Amplex red/HRP mixture. H_2O_2 was detected by the formation of the fluorescent product resorufin (460/530 nm ex/em wavelengths). The wells were read every minute and mean reading (27 values/every well/min) was considered. The first 10 min rates were calculated. Internal standard of 1 nmole H_2O_2 was added at the end of each assay. Usually no interferences were detected for all reagents used except for an increasing inhibition with GSNO at concentration higher than 1 mM.

Slow H_2O_2 releasing systems Slow rate of H_2O_2 was produced by 10–200 ng of glucose oxidase from Aspergillus Niger (E.C. 1.1.3.4) in the standard incubation medium reinforced with 10 mM glucose. H_2O_2 was measured with Amplex red/HRP as indicated above in the presence or absence of GSNO. Alternatively the effect of 100 μM GSNO was tested on 0.1 mM xanthine plus 0.13 μg/ml (0.06 mU/ml) of Xanthine oxidase as $O_2^{\cdot-}$/H_2O_2 releasing system. Reactions were conducted in a stirred vessel in a medium KPi 50 mM pH 7,4 containing 0.1 mM EDTA equilibrated in air. The rate of H_2O_2 produced was measured with Amplex red/HRP also in the presence of SOD (5 μg/ml) to dismutatate the low $O_2^{\cdot-}$ produced. In this conditions $O_2^{\cdot-}$ may account for less than 10% of the total H_2O_2 production (Fridovich 1970).

Superoxide detection Superoxide was spectrophotometrically detected at 560 nm as nitroblue tetrazolium (NBT) oxidation (0.1 mM) when superoxide was released by 3 μM phenazine methosulfate (PMS) plus 100 μM NADH (Ewing and Janero 1995) or at 320 nm as cytochrome c reduction (10 μM) when superoxide was produced by 50 μM xanthine plus 1 μU of xanthine oxidase in KPi 50 mM, 0.1 mM EDTA, pH 7.4 (Carmeli et al. 2009). 100 μM GSNO or 5 μg/ml SOD was added as indicated in the relevant figures.

Results and discussion

SNP but not GSNO increased Cplx-I H_2O_2 release

In Fig. 1 the effect of SNP (2 mM) and GSNO (0.6 mM) on the Cplx-I dependent H_2O_2 release are compared. SNP greatly stimulated H_2O_2 release from both NAD dependent substrates (Glutamate plus Malate) and Succinate in RHM. At significantly lower concentration however it did not modify H_2O_2 production (not shown). This effect is consistent with $^{\cdot}NO$ (released by mM SNP) inhibiting cytochrome oxidase and correspondingly shifting to a more reduced state the ETC similarly to CO and CN^-. The $^{\cdot}NO$

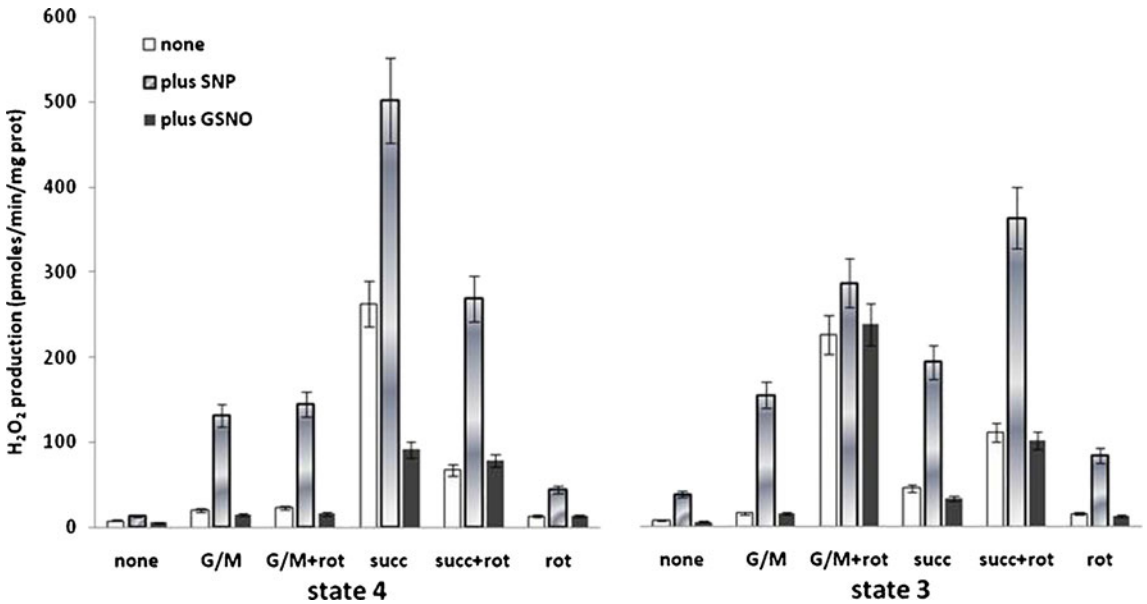

Fig. 1 Effect of SNP or GSNO on NAD-dependent substrate or succinate stimulated H_2O_2 release by RHM. RHM were incubated in the presence of the indicated substrates and inhibitors (when present glutamate was 3 mM, malate 1 mM, succinate 2 mM and rotenone 1 μM) without (State 4) or with (State 3) 1 mM ADP in the presence of Amplex red and HRP as indicated in the "Materials and methods" section. GSNO was 0.6 mM and SNP 2 mM

inhibition on cytochrome oxidase is expected to be shared by other ·NO releasing substances like GSNO. GSNO is considered a physiological ·NO buffer system at least in erythrocytes, where it can detoxify excess ·NO production by activated eNOS and act as a ·NO donor, since ·NO is slowly released upon reaction of GSNO with intracellular SH reagents (Cysteine, GSH or protein SH groups) or chemically (by DTE or DTT) (Singh et al. 1996). 0.6 mM GSNO instead strongly inhibited the succinate dependent H_2O_2 release, the inhibition being exerted without and with ADP, also in the presence of Rotenone. Similarly GSNO inhibited the slow H_2O_2 release dependent on NAD linked substrate (G/M), without and with ADP. This effect appears not to depend on ·NO inhibition of cytochrome oxidase and it is more evident at lower concentrations.

Rotenone is known to inhibit the succinate dependent H_2O_2 release and to increase the glutamate plus malate dependent release. In the presence of rotenone SNP was always stimulatory, less with the NAD dependent substrates than with succinate. A plausible explanation is that the inhibition of the cytochrome oxidase cannot modify the already highly reduced status of Cplx-I in the presence of rotenone. The rotenone inhibited release of H_2O_2 with succinate can instead be potentiated by SNP possibly increasing a release downstream of Cplx-I (cplx-III may be involved).

Since the release of ·NO by GSNO is reported to have a lag time and to be lower than that obtained with a similar amount of SNP (Ederli et al. 2009), we measured nitrite (NO_2^-) accumulated at the end of the incubation to verify that GSNO decay had occurred. Upon release ·NO is spontaneously converted in $NO_2^-+NO_3^-$; in a saline buffer system the prevalent formation of NO_2^- is expected. The corresponding increase of NO_2^- measured in mitochondria tested indeed for its dacay. The NO_2^- accumulated after 1 h accounts for 20% of added GSNO. Notably its formation appeared stimulated by succinate (the increase was significant at 10 mM GSNO (Fig. 2)).

Thus it clearly appears that the two "·NO releasing agents" behave oppositely on the same Cplx-I dependent superoxide production. The action of SNP as enhancer is in line with a more highly reduced state of the ETC components following cytochrome oxidase inhibition by ·NO. The prevailing inhibitory action of GSNO appears not dependent on ·NO release at least at low concentration.

Protein covalent modifications are possibly involved in the inhibitory effect shown by preincubation with both reagents

A covalent modification seemed instead to occur when mitochondria were preincubated with SNP. Indeed 20 min preincubation with 2 mM SNP followed by its removal,

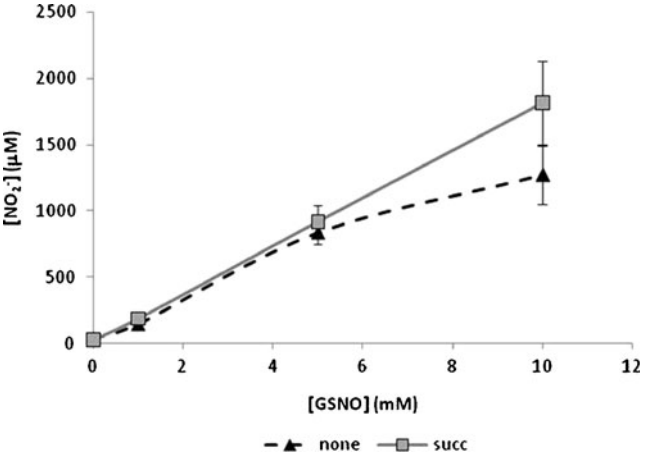

Fig. 2 Effect of succinate on nitrite detection in RHM. GSNO dacay is detected as NO_2^- formed in supernatants of RHM incubated in the presence of the indicated amount of GSNO without or with 2 mM succinate for 1 h as indicated in the "Materials and methods" section

induced an inhibition of the succinate dependent H_2O_2 release, opposite to the stimulatory effect occurring when SNP was present in the incubation (Fig. 3).

Also preincubation GSNO (10 mM plus 100 μM DTE to increase its decay) induced a similar inhibition (Fig. 4). Thus the inhibitory effect of GSNO at very high concentrations could be dependent on some covalent modification. The specie responsible is possibly ·NO since GSNO was active in the presence of DTE, which promotes the release of ·NO from GSNO.

All together these data suggest that these pretreatments may induce a covalent modification of some protein since

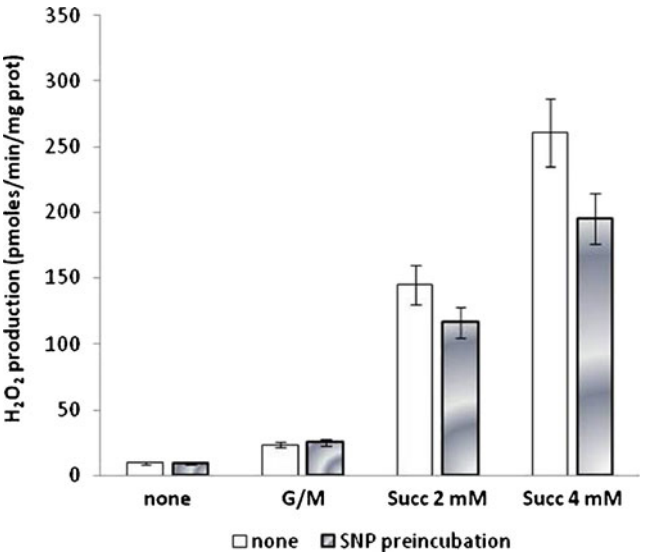

Fig. 3 Effect of preincubation with SNP on H_2O_2 release by RHM. RHM pre-incubated without (none) or with 2 mM SNP, as indicated in the "Materials and methods" section, were used for H_2O_2 detection in the same conditions of Fig. 1

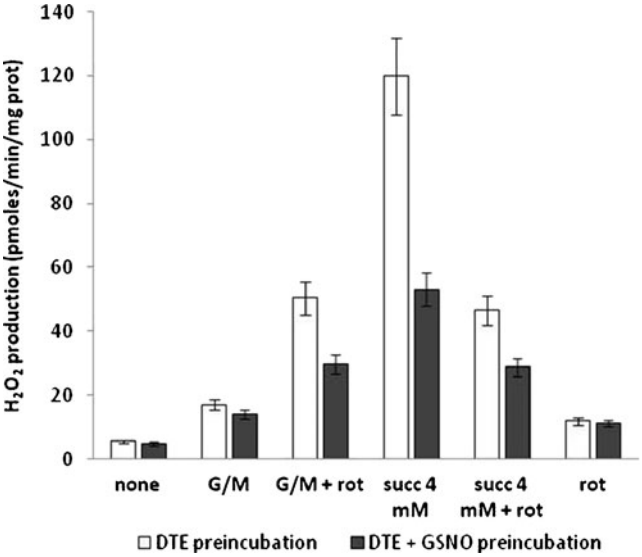

Fig. 4 Effect of preincubation with mM GSNO on the H_2O_2 release efficiency by RHM. Conditions were as in Fig. 3. SNP was substituted by 10 mM GSNO plus 100 μM DTE or DTE alone (the respective control)

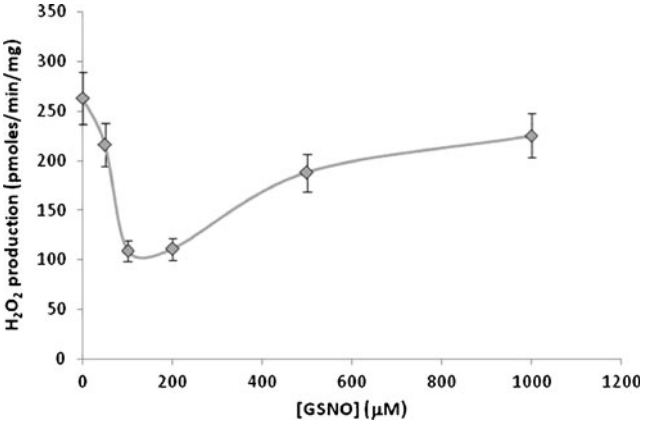

Fig. 5 Effect of GSNO on succinate stimulated H_2O_2 release by RHM. RHM were incubated in the presence of succinate (2 mM) glutamate (3 mM) and pyruvate (2 mM) and the indicated amount of GSNO. Conditions were as described in the "Materials and methods" section

the inhibition required several minutes of incubation to take place and was not rapidly relieved by removal of reagents. In these conditions the effect of the two compounds was similar and depended probably on a ·NO dependent covalent modification of some component of Cplx-I.

Presence of low GSNO concentration are always inhibitory

The effect of GSNO was then studied at the lower concentrations, where the ·NO release is undetectable as NO_2^- production. The study has been performed in mitochondria oxidizing increasing amounts of succinate in the presence of NAD dependent substrates (considered a more physiological situation). As described in previous work (Zoccarato et al. 2007, 2008, 2009; Bortolami et al. 2008), succinate induced a large release of superoxide, appreciably detected at submillimolar concentrations in brain mitochondria, and this occurred also when it was added on top of mM NAD dependent mitochondrial substrates. In heart mitochondria succinate was reactive at slightly higher concentrations.

Maximal succinate dependent H_2O_2 release was obtained with 2 mM Succinate on top of Glutamate and Pyruvate, in RHM (similar results were obtained with Glutamate and Malate). The measured H_2O_2 release was maximally inhibited by GSNO 50–200 μM (Fig. 5). At higher concentration the degree of inhibition decreased. The decreased inhibition observed at higher concentration could result from the contemporary inhibition (·NO-independent) and stimulation (·NO-dependent inhibition of cytochrome oxidase).

GSNO concentration may vary in different tissues/organs from nanomolar (200 nM is the plasmatic GSNO concentration (Bramanti et al. 2010)) to micromolar in tissues possibly differently distributed in subcellular compartmentation (Gaston et al. 2003). Isolated rat liver mitochondria have been reported to contain GSNO (Steffen et al. 2001), in an amount constituting 4% of total mitochondrial GSH. Thus, GSNO may be physiologically present in a significant micromolar range. The efficacy of GSNO as inhibitor of superoxide release at Cplx-I appears then to be relevant in a physiological concentration. We focused attention on the GSNO effect in this low range where inhibition prevails.

The inhibition by 100 μM GSNO on increasing succinate concentration added on top of glutamate plus pyruvate(G/P) is shown in the Fig. 6 for RHM. Similar results were also obtained in RBM where GSNO 20–50 μM significantly inhibited H_2O_2 production by 0.1–0.6 mM succinate added on top of G/P (data not shown).

The ·NO released by micromolar GSNO does not inhibit mitochondrial respiration

The amount of ·NO released appears not relevant at 100 μM GSNO since no clear inhibition of the mitochondrial respiration is observed (Table 1). GSNO 100 μM did not appreciably affect mitochondrial respiration in RHM with both G/P and succinate in state 3 or 4 respiration as shown in Table 1. Instead, inhibition of cytochrome oxidase, as obtained by mM SNP (or by μM CN^-), decreased respiration (50% inhibition) but increased H_2O_2 release. The effect of CN^- on H_2O_2 production was already reported in brain mitochondria. Similar effects were found in heart mitochondria. GSNO inhibited succinate dependent H_2O_2 release at all succinate concentrations and it did

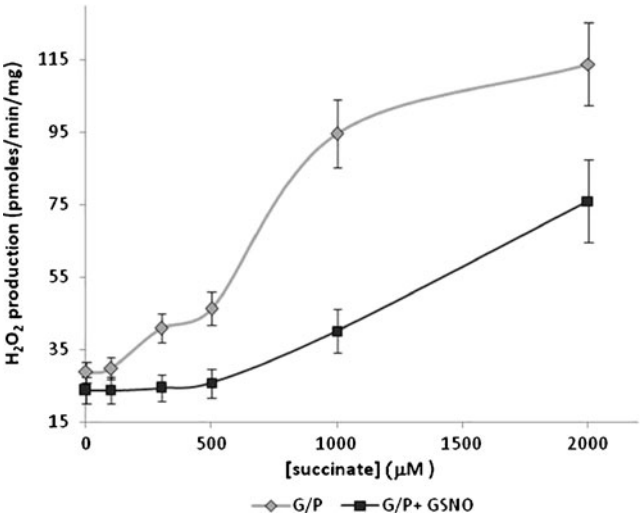

Fig. 6 Effect of GSNO on H_2O_2 release by RHM by increasing succinate concentration added on top of glutamate plus pyruvate. RHM were incubated in the presence of glutamate (3 mM) and pyruvate (2 mM) with the indicated amounts of succinate with or without 100 μM GSNO. Other conditions as described in Fig. 1

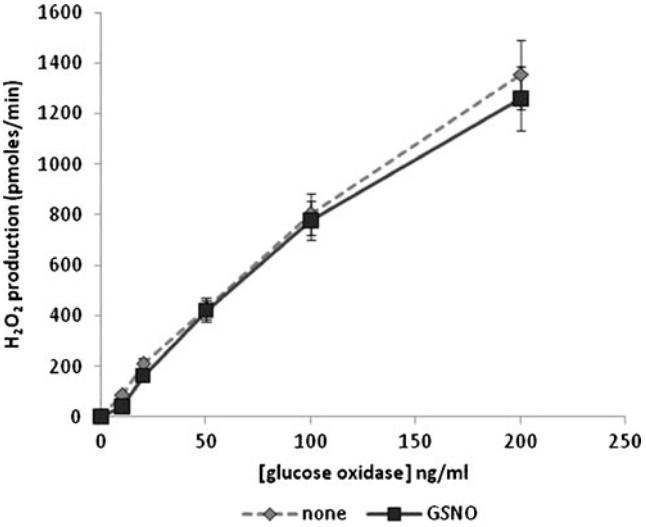

Fig. 7 Effect of GSNO (100 μM) on Amplex red/HRP detection of H_2O_2 production by the indicated low amount of glucose oxidase. The "in vitro" reaction was conducted in the same buffer of mitochondria reinforced with 10 mM glucose. Other conditions are as described in the "Materials and methods" section

not appear to change the sigmoid response to increasing succinate concentration of the curve of H_2O_2 release (Fig. 6).

GSNO at micromolar concentration does not affect the H_2O_2 detection assay

To strenghten that μM GSNO does not interfere with the HRP/Amplex red detection at low rates of H_2O_2 production, a titration curve was obtained in parallel conditions (without mitochondria) with limited amounts of glucose oxidase (10–50 ng/ml) in the presence of 10 mM glucose (Fig. 7). The rates of H_2O_2 release were similar to the rates measured in isolated mitochondria and no inhibition was observed with 100 μM GSNO. Similar results were also obtained with xanthine plus limited amount of xanthine oxidase as an $O_2^{\cdot-}/H_2O_2$ releasing system (not shown).

Table 1 Effect of GSNO, SNP or CN^- on respiration of RHM. Glutamate (3 mM) plus pyruvate (1 mM) plus succinate (2 mM) dependent O_2 consumption was detected in the oxygraph close chamber in suspensions of RHM before (state 4) and after addition of 1 mM ADP (state 3)

	O_2 consumption (nmoles/min/mg)	
	State 4	State 3
G/P+succ 1 mM	9,6±0,8	33,6±3,1
G/P+succ 1 mM+100 μM GSNO	8,7±0,6	31,8±2,8
G/P+succ 1 mM+1.5 mM SNP	4,8±0,5	15,8±0,5
G/P+succ 1 mM+30 μM CN^-	4,5±0,5	18±0,5

The inhibition by a low concentration of GSNO is a very fast process. GSNO acts suddenly, also if added after the increase of H_2O_2 production induced by succinate. The rate of H_2O_2 release continuously determined in a stirred vessel was promptly inhibited by GSNO addition (Fig. 8).

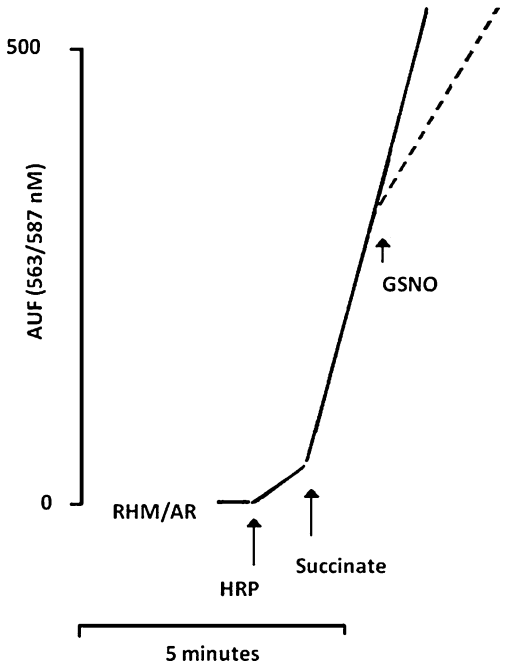

Fig. 8 Rapid inhibition of GSNO on the succinate stimulated H_2O_2 release by RHM. Reaction was followed in a stirred cuvette in a Shimatzu fluorimeter. Succinate was 0.6 mM, all other conditions as described in Fig. 1. 300 μM GSNO (*dashed line*) was added when indicated by the *arrow*. The rate of Amplex red oxidation is reported as arbitrary fluorescence units (*AUF*)

This further weakens the involvement of ·NO in the inhibition by low concentration of GSNO. Indeed: (i) GSNO decomposition is not fast (a lag time for even mM GSNO concentration is reported) while the inhibition is very rapid, (ii) it occurs at concentration where the release is very low (1 μmol/min is the maximal rate of ·NO released by 1 mM GSNO as reported in (Ederli et al. 2009)). Inhibition does not require any preincubation with GSNO. At least at the lower concentrations used (20–200 μM) the inhibition by GSNO is lost upon its removal indicating that that the effect may not be related to some protein modification. Together with the lack of interference on O_2 consumption the data suggest a direct involvement of the GSNO molecule either in modulation of the Cplx-I activity or in direct removal of the superoxide before its dismutation to H_2O_2.

The effect of GSNO at μM concentration could depend either on inhibition of superoxide release at the Cplx-I or on removal of the superoxide production prior to its dismutation to H_2O_2. GSNO does not interfere with H_2O_2 detection as shown in Fig. 7. GSNO may anyway remove $O_2^{\cdot-}$ via a direct reaction, as suggested by (Jourd'heuil et al. 1998). To confirm this possibility we performed two different tests in which the superoxide produced by the reaction of NADH plus PMS or by xanthine plus xanthine oxidase is detected as NBT oxidation or as cytochrome C reduction respectively. In both cases 100 μM GSNO exhibited a relevant inhibition: 35% in NBT oxidation (Fig. 9a) and 20% in cytochrome C reduction (Fig. 9b).

That such reaction might occur between added GSNO and intramitochondrial superoxide is not easy to prove, but it may explain part of the rapid inhibition obtained at the lowest GSNO concentration. The possible role of this reaction could be a control by GSNO of mitochondrial superoxide production. GSNO inhibits superoxide production and superoxide depletes GSNO deposits.

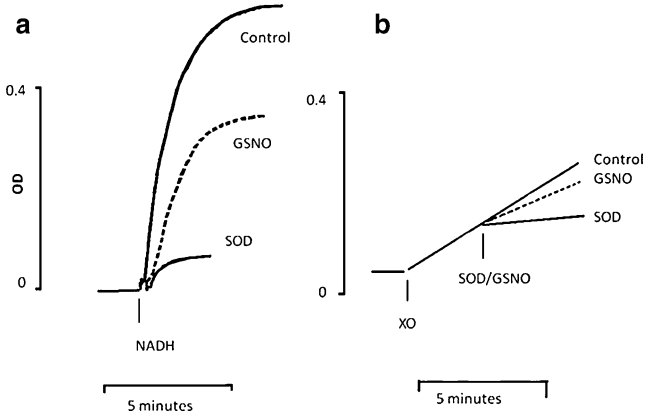

Fig. 9 Effect of GSNO (100 μM) on superoxide production by the PMS-sparked oxidation of NADH, as NBT oxidation (**a**), or by Xanthine plus Xanthine oxidase as cytochrome C reduction (**b**). The "in vitro" reaction was conducted in 100 mM Na-KPi buffer pH 7.4 as described in the "Materials and methods" section. The corresponding absorbance was followed at 560 nm for NBT oxidation or at 320 nm for cytochrome C reduction

Conclusions

SNP and GSNO, two ·NO containing compounds, act differently on Cplx-I dependent H_2O_2 production. Depending on their concentration, the two compounds may determine similar or opposite effects. When ·NO is the predominant and continuously released species, as with mM SNP, the ·NO dependent inhibition of cytochrome oxidase, evidenced by the inhibition of respiration, accounts for the increased peroxide release due to a more reduced state of the ETC components. In this case the stimulation is similar to the effect of CN^- (Zoccarato et al. 2009). CO may probably have the same effect. An inhibitory effect on H_2O_2 production is instead exhibited following preincubation and removal from the mitochondrial suspension of 10 mM GSNO plus DTE, or of 2 mM SNP. The similarity of the effect may require a common mechanism of action, possibly an inhibitory modification (S-nitrosylation) of some component of Cplx-I. This effect was evident after washout of SNP and GSNO.

At μM concentration GSNO, but not SNP, greatly decreased mitochondrial H_2O_2 release induced either by succinate, by NAD-dependent substrates or both. This is very interesting because it occurs both in rat heart and brain mitochondria suggesting a general phenomenon involving physiological GSNO concentrations (μM GSNO was found in liver mitochondria (Steffen et al. 2001)). This inhibition is not due to interferences with the H_2O_2 detection system used. It is not dependent on ·NO, since respiration is not affected. A negative modulation of Cplx-I superoxide production is likely exerted by GSNO. It may, at least partly, depend on the ability of GSNO to react with and to remove superoxide. The inhibitory effect on succinate dependent H_2O_2 release at Cplx-I occurs suddenly following GSNO addition. Also the GSNO decay rate, measured as NO_2^- production, is increased when succinate is present. Both events indicate that a reaction of GSNO with superoxide may occur in mitochondria.

A strict interplay between NOS-dependent ·NO production and ROS, acting in general in an opposite way, is well documented. Thus GSNO present in vivo may down regulate ROS production by mitochondrial Cplx-I. GSNO is known to prevent ischemic damage (Lima et al. 2009; Khan et al. 2009) and brain trauma (Khan et al. 2009). Thus physiological GSNO concentration may down regulate superoxide production and be depleted by a sustained superoxide production.

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