Mitochondrial Nitric-oxide Synthase Stimulation Causes Cytochrome c Release from Isolated Mitochondria

EVIDENCE FOR INTRAMITOCHONDRIAL PEROXYNITRITE FORMATION*

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Pedram Ghafourifar‡, Ursula Schenk, Sabine D. Klein, and Christoph Richter

From the Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zurich, Switzerland

Nitric oxide (NO) is synthesized by members of the NO synthase (NOS) family. Recently the existence of a mitochondrial NOS (mtNOS), its Ca\(^{2+}\) dependence, and its relevance for mitochondrial bioenergetics was reported (Ghafourifar, P., and Richter, C. (1997) FEBS Lett. 418, 291–296; Giulivi, C., Poderoso, J. J., and Boveris, A. (1998) J. Biol. Chem. 273, 11038–11043). Here we report on the possible involvement of mtNOS in apoptosis. We show that uptake of Ca\(^{2+}\) by mitochondria triggers mtNOS activity and causes the release of cytochrome c from isolated mitochondria in a Bel-2-sensitive manner. mtNOS-induced cytochrome c release was paralleled by increased lipid peroxidation. The release of cytochrome c as well as increase in lipid peroxidation were prevented by NO inhibitors, a superoxide dismutase mimic, and a peroxynitrite scavenger. We show that mtNOS-induced cytochrome c release is not mediated via the mitochondrial permeability transition pore because the release was aggravated by cyclosporin A and abolished by blockade of mitochondrial calcium uptake by ruthenium red. We conclude that, upon Ca\(^{2+}\)-induced mtNOS activation, peroxynitrite is formed within mitochondria, which causes the release of cytochrome c from isolated mitochondria, and we propose a mechanism by which elevated Ca\(^{2+}\) levels induce apoptosis.

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Nitric oxide (NO) is a molecule of prime importance in biology. The most-cited and best understood physiological target for NO is the heme-containing protein, soluble guanylyl cyclase (1). However, at physiological concentrations, NO also binds to another hemoprotein, cytochrome oxidase (COX), the terminal enzyme of the mitochondrial respiratory chain, and thereby controls cellular functions via reversible inhibition of respiration (reviewed in Ref. 2). NO is synthesized by members of the NO synthase family (NOS, EC 1.14.13.39; reviewed in Ref. 3). In 1997, we reported for the first time on the presence of a constitutively expressed and continuously active NOS in mitochondria (mtNOS), its localization in the inner mitochondrial membrane, its Ca\(^{2+}\) dependence, and that the enzyme exerts substantial control over mitochondrial respiration and mitochondrial transmembrane potential (\(\Delta \psi \)) (4). Soon thereafter the presence of mtNOS and its localization were confirmed, and the enzyme was enriched and shown to cross-react with antibodies directed against inducible NOS (5).

Apoptosis, also called programmed cell death, is an evolutionary conserved phenomenon which regulates normal cellular turnover. Mitochondria are essential for at least certain forms of apoptosis (reviewed in Ref. 6). For example, cytochrome c, a mitochondrial protein that is part of the respiratory chain, triggers apoptosis once it is dislocated from the organelle. It is now well accepted that many factors drive cells into apoptotic death via mitochondrial cytochrome c release.

NO reacts with O\(_2^-\) to produce the powerful oxidizing agent, peroxynitrite (ONOO\(^-\)). Recently many studies focused on the role of NO and ONOO\(^-\) in apoptosis (7–10). Their exact source(s) and the mechanism(s) are, however, not yet fully elucidated. An increase in cytosolic Ca\(^{2+}\) level caused by, e.g. glutamate receptor stimulation, is apoptogenic (reviewed in Ref. 11). Many recent reports show that mitochondrial Ca\(^{2+}\) uptake is an essential step in Ca\(^{2+}\)-induced apoptosis (12–14). This kind of programmed cell death is accompanied by increased NO activity (12, 13) and prevented by mitochondrial superoxide dismutase (SOD), MnSOD (10, 15), or by the ONOO\(^-\) scavenger, urate (16). Mitochondria produce NO in a Ca\(^{2+}\)-dependent manner (4) and are a rich source of O\(_2^-\) (17). Therefore, intramitochondrial Ca\(^{2+}\)-dependent ONOO\(^-\) formation seems likely. Here we show that upon Ca\(^{2+}\) uptake by isolated mitochondria, mtNOS is stimulated and cytochrome c is released in a Bel-2-sensitive manner. We provide evidence that the observed cytochrome c release is due to intramitochondrial ONOO\(^-\) formation because it is prevented by NO inhibitors, an SOD mimic, and an ONOO\(^-\) scavenger. We suggest that Ca\(^{2+}\)-induced apoptosis is at least partly mediated via mtNOS.

**EXPERIMENTAL PROCEDURES**

N\(^{\text{V}}\)-monomethyl-L-arginine (L-NMMA), N\(^{\text{3}}\)-(3-aminomethylbenzyl) acetamide (1400W), and manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). Horse heart cytochrome c and L-[2,3-H]arginine (36.8 Ci/mmol) were from Sigma, mouse mononuclear cytochrome c antibody from BD (Bedford, NJ), anti-mouse Ig, horseradish peroxidase from Amersham Pharmacia Biotech (Dübendorf, Switzerland), 6-His-human Bel-2 from Novartis (Basel, Switzerland), and L-[\text{ureido-}^{14}\text{C}]citrulline (58.8 mCi/mmol) from NEN Life Science Products.

**Mitochondrial Preparation**—Isolation of rat liver mitochondria was performed by differential centrifugation as described (17). The protein content of mitochondria and the mitochondrial supernatants were determined by the Biuret method with bovine serum albumin as standard.

**Detection of Cytochrome c Release**—Fresly isolated mitochondria (50 mg of protein/ml) were incubated at 4 °C in 0.1 M HEPES buffer, pH 7.1, containing protease inhibitors: aprotinin, pepstatin A, phenylmethanesulfonyl fluoride, and leupeptin (2 μg/ml each). NOS inhibitors,
mtNOS Releases Cytochrome c

Fig. 1. mtNOS stimulation induces cytochrome c release. Mitochondria were incubated in the presence of 5 μM rotenone and 0.8 mM succinate except in panel D as indicated. A, stimulation of mtNOS (mtNOS stim) was performed by 100 μM Ca²⁺, 100 μM L-arginine, and 12 μM BH₄. L-NMMA (10 mM), 1400W (100 μM), Bcl-2 (8 pmol/mg of mitochondrial protein), MnTBAP (10 μM), or urate (100 μM) was present where indicated (for details, see "Experimental Procedures"). B, Ca²⁺, L-arginine, and BH₄ (concentrations as in panel A) were added in combination or alone. L-NMMA or Bcl-2 were present as in panel A. C, concentration dependence of Ca²⁺-induced cytochrome c release and the effect of CSA (1 μM) on cytochrome c release induced by 1 μM Ca²⁺. L-NMMA was present as in panel A. D, the release of cytochrome c induced by 10 μM Ca²⁺ when mitochondrial Ca²⁺ uptake was blocked by 8 μM ruthenium red (RR) or when mitochondria were de-energized by 50 nM antimycin A plus 1.7 μg of oligomycin/mg of mitochondrial protein (AA + OM), or by omitting succinate (Succ +).

L-NMMA (10 mM) or 1400W (100 μM), 6-His-human Bcl-2 (8 pmol/mg of mitochondrial protein) (18), SOD mimic, MnTBAP (10 μM), or peroxynitrite scavenger, urate (100 μM) (16) were also present in the incubation buffer as indicated. After 10 min of incubation, 100 μM Ca²⁺, 100 μM L-arginine, and 12 μM tetrahydrobiopterin (BH₄) from a 1.2 mM stock solution prepared instantaneously before the experiment (4) were added, and the mitochondrial suspensions were incubated for 5 min at room temperature. Mitochondrial respiration was then supported by 0.8 mM K⁺-succinate in the presence of 5 μM rotenone for 10 min at room temperature. Samples were spun at 12,000 × g for 10 min at 4 °C, and the resulting supernatants were spun at 100,000 × g for 15 min at 4 °C. The supernatants of the second centrifugation were used for the detection of cytochrome c by Western blot analysis as described (18). Cyclesporin A (CSA, 1 μM) or mitochondrial Ca²⁺ uptake blocker ruthenium red (RR; 8 μM) were added before Ca²⁺. Mitochondrial de-energization was performed by addition of 50 nM antimycin A to block complex III and 1.7 μg of oligomycin/mg of mitochondrial protein to block ATPase before succinate (18) or by omitting the respiratory substrate.

Determination of Mitochondrial Lipid Peroxidation (LPO)—Samples were prepared as described above, except that mitochondria were incubated at 2 mg/ml and in the absence of protease inhibitors. Mitochondrial LPO was determined by thiobarbituric acid assay as described (19).

Determination of mtNOS Activity—Samples were prepared as described above except that mitochondria were incubated at 10 mg/ml and in the absence of protease inhibitors. mtNOS activity was determined by measurement of the conversion of L-[³H]arginine to L-[³H]citrulline as described (20) and is expressed as cpn/mg of mitochondrial protein.

RESULTS AND DISCUSSION

mtNOS stimulation induces cytochrome c release from isolated mitochondria in a manner that is prevented by two NOS inhibitors, L-NMMA and 1400W (Fig. 1A). This panel also shows that Bcl-2 prevents mtNOS-induced cytochrome c release, which indicates that the release is not because of a general mitochondrial damage followed by a nonspecific protein release, but it is a specific phenomenon relevant to apoptosis. A considerable number of recent reports show that endogenously formed NO induces Bcl-2-sensitive apoptosis (10, 13, 15, 21–24), which is accompanied by mitochondrial dysfunction (10, 13), increased LPO (10, 24, 25). It is also well demonstrated that increased cytosolic Ca²⁺-induced apoptosis requires mitochondrial Ca²⁺ uptake (12–14), is paralleled by increased NOS activity (12, 13, 26), and is prevented by lowering the mitochondrial O₂⁻ level by MnSOD (10, 15) or by scavenging ONOO⁻ with urate (16). The reaction of NO and O₂⁻ with the rate constant of 1.9 × 10¹⁰ M⁻¹ s⁻¹ (27) is one of the fastest reactions known in biology. Mitochondria produce NO in a Ca²⁺-dependent fashion (4), and they are well known sources of O₂⁻ radicals. Intramitochondrial Ca²⁺-dependent ONOO⁻ formation is, therefore, very likely. Fig. 1A shows that mtNOS-induced cytochrome c release is prevented by the SOD mimic, MnTBAP, as well as by the ONOO⁻ scavenger, urate. This finding strongly suggests that ONOO⁻ is indeed formed within mitochondria and that it releases mitochondrial cytochrome c. This may explain the mechanism by which an elevated Ca²⁺ level induces apoptosis in a manner that requires mitochondrial Ca²⁺ uptake and is prevented by inhibiting NOS activity, lowering O₂⁻ level, and scavenging ONOO⁻.

It is known that ONOO⁻ induces LPO (10, 28, 29). Fig. 2 shows that, upon mtNOS stimulation, LPO is increased in a manner that is sensitive to L-NMMA, Bcl-2, MnTBAP, and...
III and IV, complex III remains mostly reduced, and therefore, hierarchically arranged mitochondrial respiratory complexes the uptake of Ca\(^{2+}\) chondria (34). There is also evidence for the presence of BH4 of mitochondrial protein), or L-NMMA (10 mM) was present from the beginning of the measurement.

The mitochondrial uncoupler, carbonyl cyanide m-cresol cyanide (35) and calmodulin (36, 37) in mitochondria.

To address the mechanism by which Bcl-2 prevents mtNOS-induced cytochrome \(c\) release, we measured mitochondrial Ca\(^{2+}\) uptake by RR prevents Ca\(^{2+}\)1 uptake (not shown). Additionally, we measured mitochondrial release and uptake blockers. Fig. 1C shows that uptake of Ca\(^{2+}\) by respiring mitochondria causes a drastic fall in \(\Delta \Psi\) that

urate. This figure also shows that exogenously added cytochrome \(c\) prevents increased LPO. From the elegant study by Cai and Jones (30) it is known that cytochrome \(c\) release is the cause and not the consequence of reactive oxygen species formation in mitochondria. We have recently confirmed this finding by showing that the decreased mitochondrial \(O_2\) consumption, \(\Delta \Psi\) and Ca\(^{2+}\) retention, consequent to ceramide-induced mitochondrial cytochrome \(c\) loss, are recovered by addition of exogenous cytochrome \(c\) (18). Very recently, it has also been reported that both the shape and the volume alterations of mitochondria because of cytochrome \(c\) loss are reversible (31).

Upon release of cytochrome \(c\) from its native location within the hierarchically arranged mitochondrial respiratory complexes III and IV, complex III remains mostly reduced, and therefore, electrons become available for \(O_2^-\) formation. Prevention by Bcl-2 of cytochrome \(c\) release retains the possibility for electrons to flow from complex III to cytochrome \(c\), and from there to complex IV and, consequently, decreases the availability of electrons for the formation of \(O_2^-\), one of the two precursors of ONOO\(^-\).

In the experiments reported above, Ca\(^{2+}\), L-arginine, and BH\(_4\) were provided to mitochondria. Fig. 1B shows that Ca\(^{2+}\) per se is sufficient to trigger cytochrome \(c\) release in an \(\Delta \Psi\) and Bcl-2 sensitive manner and Fig. 1C shows that the effect of Ca\(^{2+}\) is concentration-dependent. It is not surprising that Ca\(^{2+}\) per se is sufficient for mtNOS-induced cytochrome \(c\) release, because other substrate/cofactors seem to be available in mitochondria in adequate concentrations. Intramitochondrial concentrations of L-arginine (32) and NADPH (33) are in the mM range. FAD and FMN are components of mitochondrial respiratory complexes I and II and, therefore, present in mitochondria (34). There is also evidence for the presence of BH\(_4\) (35) and calmodulin (36, 37) in mitochondria.

To establish that the observed cytochrome \(c\) release requires the uptake of Ca\(^{2+}\) into the mitochondria, we used specific mitochondrial release and uptake blockers. Fig. 1C shows that sequestration of Ca\(^{2+}\) within mitochondria by CSA, a compound known to block the specific mitochondrial Ca\(^{2+}\) release pathway (38), aggravates mtNOS-induced cytochrome \(c\) release in an \(\Delta \Psi\) sensitive manner. CSA is also reported to be a closure of the nonspecific solute transport across the inner mitochondrial membrane, the mitochondrial permeability transition pore, which is considered to be the reason for many features of apoptosis including cytochrome \(c\) release (reviewed in Ref. 39). Because CSA further increases the release of cytochrome \(c\) induced by mtNOS stimulation (Fig. 1C), we conclude that mtNOS-induced cytochrome \(c\) release is not mediated via the mitochondrial permeability transition. Blockade of mitochondrial Ca\(^{2+}\) uptake by RR prevents Ca\(^{2+}\) induced cytochrome \(c\) release (Fig. 1D). This finding is compatible with reports by other investigators that RR prevents apoptosis induced by elevated cytosolic Ca\(^{2+}\) levels (12, 14, 40, 41). Also, when mitochondria were de-energized by antimycin A plus oligomycin or by omitting succinate and therefore did not take up Ca\(^{2+}\), mtNOS activity was decreased (Fig. 3A) and cytochrome \(c\) release was prevented (Fig. 1D). This finding also confirms recent reports (18, 31) that cytochrome \(c\) is not detached from the mitochondrial inner membrane because of a fall in mitochondrial transmembrane potential, e.g. upon uptake of Ca\(^{2+}\).

To address the mechanism by which Bcl-2 prevents mtNOS-induced cytochrome \(c\) release, we measured mitochondrial Ca\(^{2+}\) uptake (dual wavelength spectroscopy using Arsenazo III as the probe) (18) and observed that Bcl-2 does not decrease mitochondrial Ca\(^{2+}\) uptake (not shown). Additionally, we measured mtNOS activity and observed that Bcl-2 does not decrease it (Fig. 3A). Fig. 3B shows that Ca\(^{2+}\) induced decreased mitochondrial \(O_2\) consumption is prevented by \(\Delta \Psi\), but not by Bcl-2. Also Fig. 3C shows that uptake of Ca\(^{2+}\) by respiring mitochondria causes a drastic fall in \(\Delta \Psi\) that
demonstrate that decreased O$_2$ consumption and ∆Ψ induced by mitochondrial Ca$^{2+}$ uptake is because of NO formation by mtNOS, and not cytochrome c release, and that prevention by Bcl-2 of mtNOS-induced cytochrome c release is not because of a decreased mtNOS activity. In contrast, mitochondrial de-energization by antimycin A prevents mtNOS-induced cytochrome c release (Fig. 1D) because of a drastic decrease in mtNOS activity (Fig. 3A).

Altogether, these results show that uptake of Ca$^{2+}$ by mitochondria followed by mtNOS stimulation causes mitochondrial cytochrome c release and increased LPO, and provide evidence that these events are mediated via intramitochondrial ONOO$^-$/ formation. We propose that mtNOS plays a hitherto undetected role in apoptosis induced by elevated cytosolic Ca$^{2+}$ levels.

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REFERENCES
1. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) Pharmacol. Rev. 43, 109–142
2. Ghafourifar, P., and Richter, C. (1999) in From Symbiosis to Eukaryotism: Endocytobiology VII (Wagner, E., ed) pp. 503–516, Geneva University Press
3. Knowles, R. G., and Moncada, S. (1994) Biochem. J. 298, 249–258
4. Ghafourifar, P., and Richter, C. (1997) FEBS Lett. 418, 291–296
5. Guilivi, C., Pederoso, J. J., and Boveris, A. (1998) J. Biol. Chem. 273, 11038–11043
6. Green, D. R., and Reed, J. C. (1997) Science 281, 1305–1312
7. Lin, K. T., Yue, J. Y., Nomen, M., Spur, B., and Wong, P. Y. K. (1995) J. Biol. Chem. 270, 16487–16490
8. Lin, K. T., Yue, J. Y., Lin, M. C., Spokas, E. G., Sun, F. F., and Wong, P. Y. K. (1996) Am. J. Physiol. 274, C655–C660
9. Szabo, C., Cuzzocrea, S., Zingarelli, B., O’Connor, M., and Salzman, A. L. (1997) J. Clin. Invest. 100, 723–735
10. Keller, J. N., Kindy, M. S., Holtsberg, F. W., St Clair, D. K., Yen, H. C., Germeyer, A., Steiner, S. M., Bruce-Keller, A. J., Hutchins, J. B., and Mattson, M. P. (1998) J. Neurosci. 18, 687–697
11. McConkey, D. J., and Orrenius, S. (1997) Biochem. Biophys. Res. Commun. 239, 357–366
12. Stout, A., Raphael, H. M., Kantererwicz, B. I., Klann, E., and Reynolds, I. J. (1998) Nat. Neurosci. 1, 366–373
13. Almeida, A., Heales, S. J. R., Bolanos, J. P., and Medina, J. M. (1998) Brain Res. 790, 209–216
14. Kruman, I. I., and Mattson, M. P. (1999) J. Neurochem. 72, 529–540
15. Gonzalez-Zulutegui, M., Ensz, L. M., Mukhina, G., Lebovitz, R. M., Zwaacka, R. M., Engelhardt, J. F., Oberley, L. W., Dawson, V. L., and Dawson, T. M. (1998) J. Neurosci. 18, 2040–2055
16. Kruman, I., Guo, Q., and Mattson, M. P. (1998) J. Neurosci. Res. 51, 293–308
17. Lötscher, H. R., Winterhalter, K. H., Carafoli, E., and Richter, C. (1980) J. Biol. Chem. 255, 9325–9330
18. Ghafourifar, P., Klein, S. D., Schuch, R., Schenk, U., Rocha, S., Pruschky, M., and Richter, C. (1999) J. Biol. Chem. 274, 6080–6084
19. Klein, S. D., Wilt, H., and Richter, C. (1997) Arch. Biochem. Biophys. 348, 313–319
20. Richter, C., Schweizer, M., and Ghafourifar, P. (1999) Methods Enzymol. 311, 381–393
21. Estevez, A. G., Spear, N., Manuel, S. M., Radi, R., Henderson, C. E., Barbeito, L., and Beckman, J. S. (1998) J. Neurosci. 18, 923–931
22. Brune, B., Gota, C., Meidler, U. K., Sandau, K., Hirvonen, M., and Lapetina, E. G. (1997) J. Biol. Chem. 272, 7253–7258
23. Messmer, U. K., Reimer, D. M., Reed, J. C., and Brune, B. (1996) FEBS Lett. 384, 162–166
24. Ferrante, R. J., Hantraye, P., Brouillet, E., and Beal, M. F. (1999) Brain Res. 823, 177–182
25. Leist, M., Volbracht, C., Kuhnle, S., Fava, E., Ferrando-May, E., and Nicotera, P. (1997) Mol. Med. 3, 750–764
26. Le, W. D., Colon, L. V., Xie, W. J., Smith, R. G., Alexianu, M., and Appel, S. H. (1995) Brain Res. 686, 49–60
27. Kissner, R., Nauer, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997) Chem. Res. Toxicol. 10, 1285–1292
28. Radi, R., Beckman, J. S., Bush, M. K., and Freeman, B. A. (1991) Arch. Biochem. Biophys. 288, 481–487
29. Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyunaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994) J. Biol. Chem. 269, 26066–26075
30. Cai, J., and Jones, D. P. (1998) J. Biol. Chem. 273, 11401–11404
31. Martinou, I., Desagher, S., Eskes, R., Antonsson, B., Andre, E., Fakan, S., and Martinou, J. C. (1999) J. Cell Biol. 144, 883–889
32. Dolinskaya, M., and Albrecht, J. (1998) Neurochem. Int. 33, 233–236
33. Williamson, J. R., and Corkey, B. E. (1979) Methods Enzymol. 55, 200–222
34. Darley-Umar, V., Ragan, I., Smith, P., and Wilson, M. (1994) in Mitochondria: DNA, Proteins, and Diseases (Darley-Umar, V., and Schapira, A. H. V., eds) pp. 1–25, Portland Press, UK
35. Rembold, H., and Buff, K. (1972) Eur. J. Biochem. 28, 586–591
36. Hatae, O., Doi, A., Itano, T., Matsui, H., and Ohmura, Y. (1985) Biochem. Biophys. Res. Commun. 132, 63–66
37. Itano, T., Matsui, H., Doi, A., Ohmura, Y., and Hatae, O. (1986) Biochem. Int. 15, 787–792
38. Richter, C., Theus, M., and Schlegel, J. (1990) Biochem. Pharmacol. 40, 779–782
39. Hirsch, T., Marzo, I., and Kroemer, G. (1997) Biosci. Rep. 17, 67–76
40. Baek, J. H., Lee, Y. S., Kang, C. M., Kim, J. A., Kwon, K. S., Son, H. C., and Kim, K. W. (1997) Int. J. Cancer 73, 725–729
41. Kruman, I. I., Nath, A., and Mattson, M. P. (1998) Exp. Neurol. 154, 276–288