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Specific nitration of tyrosines 46 and 48 makes cytochrome *c* assemble a non-functional apoptosome

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A B S T R A C T

Under nitroxidative stress, a minor fraction of cytochrome c can be modified by tyrosine nitration. Here we analyze the specific effect of nitration of tyrosines 46 and 48 on the dual role of cytochrome c in cell survival and cell death. Our findings reveal that nitration of these two solvent-exposed residues has a negligible effect on the rate of electron transfer from cytochrome c to cytochrome c oxidase, but impairs the ability of the heme protein to activate caspase-9 by assembling an non-functional apoptosome. It seems that cytochrome c nitration under cellular stress counteracts apoptosis in light of the small amount of modified protein. We conclude that other changes such as increased peroxidase activity prevail and allow the execution of apoptosis.

Keywords:
Apoptosome
Caspase-9 activation
Cytochrome *c*
Cytochrome *c* oxidase
Electron transfer
Mitochondrial respiration
Post-translational modification
RNOS
Tyrosine nitration

1. Introduction

Living cells obtain energy through oxidative phosphorylation, or mitochondrial respiration, which involves the transfer of electrons from NADH and $FADH_2$ to oxygen and the subsequent synthesis of ATP. The incomplete oxygen reduction leads to the formation of intermediate radicals, the so-called reactive nitrogen and/or oxygen species (RNOS) [1–3], which are usually eliminated by cellular detoxifying systems. Such mechanisms may fail during cell aging or under stress conditions, thereby increasing RNOS concentration.

One of the most deleterious reactive species is the strong oxidant peroxynitrite, which is formed by reaction between superoxide anion and nitric oxide. Peroxynitrite serves as an in vivo nitrating agent [4] that mainly promotes nitration of tyrosines in mitochondrial proteins [5–8], but its lifetime is long enough to cross the membrane and react with biomolecules in other compartments [5,6]. Actually, the cumulative protein tyrosine nitration might be responsible for alterations in protein function, turnover and localization, with the concomitant implication in the pathogenesis of diseases [9–13] undergoing nitroxidative stress.

Respiratory cytochrome *c* (C*c*) is one of the main targets for RNOS – and, in particular, for peroxynitrite – in mitochondria, where the heme protein is both nitrated and nitrosylated [14,15]. Under homeostasis, C *c* acts as an electron shuttle between the cytochrome bc_1 and cytochrome *c* oxidase (C *c*O) membrane-embedded complexes [16]. However, the pro-apoptotic stimuli make C *c* bind to and oxidize the mitochondria-specific phospholipid cardiolipin (CL) [17], which in turn allows the translocation of C *c* into the cytoplasm so as to trigger the apoptosis signalling pathway upon binding to the apoptosis protease-activation factor (Apaf-1) and apoptosome assembly [18,19]. In vitro nitration of human C *c* tyrosine residues at positions 67, 74 and 97 impairs the two antagonist functions of C *c* in cell life (respiration) and cell death (apoptosis) [20,21], in agreement with previous data obtained in vivo with Tyr67-nitrated C *c* [22]. In contrast, tyrosine nitration can increase the peroxidase activity of C *c* [21,23,24], an example of gain-of-function

Abbreviations: Ac-LEHD-AFC, N-acetyl-Leu-Glu-His-Asp-(7-amino-4-trifluoromethyl coumarin); Apaf-1, apoptosis protease-activating factor-1; CL, cardiolipin; Cc, cytochrome *c*; CcO, cytochrome *c* oxidase; PC9, pro-caspase 9; RNOS, reactive nitrogen/oxygen species

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modification that sheds light to the biological significance of nitration since a small fraction of nitrated Cc may be sufficient to elicit a substantive biological signal.

Most physicochemical and functional studies of Cc have been performed with the horse protein modified by tyrosine chemical nitration at positions 67, 74 and 97 [20–27]. Significant differences are observed depending on which ever the nitrated residue is. Actually, previous reports describe how nitration of Tyr74 modulates the Cc functions, whereas nitration of Tyr97 has no any functional effect [20,21].

In the case of Tyr46 and Tyr48 of human Cc, which are solvent-exposed and easily nitrated in vitro [28], the mechanism by which the $-NO_2$ radical alters the Cc functions remains unclear. Here we have designed two human Cc mutants with all but one of their tyrosine residues – at position 46 or 48 – replaced by phenylalanines. Our experimental data demonstrate that in vitro nitration of either Tyr48 (which is a highly conserved residue in all organisms) or Tyr46 (which is only present in human and plant Cc) leads to the assembly of a non-functional apoptosome, which fails in caspases activation.

2. Materials and methods

2.1. Sample preparation

Recombinant human respiratory Cc, either the WT species or the monotyrosine mutants in which only Tyr46 or Tyr48 is present (the hereincalled *h*-Y46 or *h*-Y48 variants), were expressed in *Escherichia coli* DH5α strain and further purified by ionic exchange chromatography, as previously described [20,21]. Peroxynitrite synthesis and nitration of monotyrosine Cc mutants were performed as previously described [20,21,28] with the following modifications: Fe^{3+}-EDTA concentration and the number of peroxynitrite additions were increased up to 1.5 mM and 10 bolus additions, respectively. The nitration reaction was performed under acidic conditions (pH 5.0).

The nitrated Cc species were intensively washed in 10 mM potassium phosphate at pH 6 and purified to 95% homogeneity, as reported in Ref. [20]. Purity of nitrated Cc preparations, as well as molecular mass and specific nitrated tyrosine of each mutant, were confirmed by tryptic digestion and MALDI-TOF (Bruker-Daltonics, Germany) analyses. Western Blotting Solution (Amersham) with antibodies anti-nitrotyrosine (Biotem) was used to confirm the presence of $-NO_2$ groups in the Cc samples upon nitration. Samples were concentrated to 0.2–2.0 mM in 5 mM sodium phosphate buffer (pH 6). The pyridine hemochrome assay was used to estimate the extinction coefficients of the nitrated and non-nitrated forms of monotyrosine mutants [29]. When oxidation of methionine residues was detected, the samples were discarded.

Recombinant human Apaf-1 was expressed and purified as described in Refs. [30,31]. Recombinant pro-caspase 9 (PC9) was produced and purified as in Ref. [21]. Horse cytochrome *c* oxidase (CcO) was purified as reported in Ref. [20]. CcO concentration was estimated by using a differential extinction coefficient $\Delta\varepsilon_{604-630}$ of 17 mM^{-1} cm^{-1} for the reduced *minus* oxidized protein [32].

2.2. Kinetic analysis

The kinetics of electron transfer from the non-nitrated and nitrated Cc species to horse CcO were analyzed by laser flash spectroscopy by following the absorbance change at 550 nm. The redox reactions were induced by EDTA-photoreduced FMN, as previously reported [20]. All experiments were performed under pseudo-first order conditions, with the concentration of oxidized CcO well exceeding that of reduced Cc per flash. Further kinetic analyses were carried out to estimate the bimolecular rate constant (k_2) for the nitrated and non-nitrated mutants, as well as the association (K_A) and effective electron transfer rate (k'_{et}) constants for WT Cc [20].

2.3. Apaf-1/Cc cross-linking, light scattering and caspase-9 activation

To detect the interaction between Cc and Apaf-1 in *Jurkat* T cell extracts, the cross-linking, light scattering and caspase-9 assays were run as described in Ref. [21].

3. Results

3.1. Nitration of monotyrosine mutants of Cc

Nitrated monotyrosine Cc mutants in which only Tyr46 or Tyr48 is present were separated from non-nitrated protein in a CM-cellulose column equilibrated with 1.5 mM borate, pH 9.0, using a 0–100 mM NaCl gradient. Nitrated Cc eluted at a much lower salt concentration than native protein because of the extra negative charge of deprotonated tyrosyl anions, whose pK_a is modified by the strong electron-withdrawing effect of the substituent $-NO_2$ group at the 3-position [24]. The purity to homogeneity of nitrated Cc preparations was corroborated by SDS–PAGE and Western Blot using antibodies anti-nitrotyrosine (Biotem) to detect the presence of the $-NO_2$ group (Fig. 1). In addition, the molecular mass and the specifically nitrated tyrosine residue of each mutant were confirmed by tryptic digestion and MALDI-TOF (Bruker-Daltonics, Germany) analyses, as recently reported [28].

3.2. Electron transfer between oxidized Cc mutants and CcO

Cc serves as a one-electron carrier between cytochrome bc_1 complex and CcO at the end of the mitochondrial electron transport chain. In a previous report [20], the kinetics of horse CcO reduction by the nitrated and non-nitrated species of the *h*-Y67, *h*-Y74 and *h*-Y97 mutants were studied by laser flash spectroscopy. Here, we have analyzed the effect of nitration of Cc at positions 46 and 48 using the *h*-Y46 and *h*-Y48 variants to reduce CcO. As can be seen in Fig. 2, WT Cc shows a non-linear dependence of the observed pseudo-first-order rate constant (k_{obs}) upon Cc CcO concentration at pH 6.5, thus indicating the formation of a kinetically detectable transient Cc–CcO electron transfer complex, as previously observed at pH 7.5 [20]. However, the k_{obs} values at pH 6.5 with the non-nitrated and nitrated forms of *h*-Y46 and *h*-Y48 show in all cases a linear dependence on Cc CcO concentration. This suggests that electron transfer is much faster than complex dissociation, in agreement with a collisional reaction mechanism [33]. The resulting values for the bimolecular rate constant (k_2) estimated with the two Cc mutants show that nitration slightly decreases the ability of *h*-Y46 to donate electrons to Cc CcO and has an even lower effect on *h*-Y48 (Table 1). At pH 7.5, the effect of nitration on the k_2 values with *h*-Y46 and *h*-Y48 is practically negligible (not-shown).

3.3. Cc-dependent activation of caspases

To check how the nitration of Cc alters the apoptotic process, the apoptosome was first reconstituted in vitro by incubating recombinant Apaf-1 with either the nitrated or non-nitrated species of *h*-Y46 and *h*-Y48. The subsequent addition of PC9 allowed to follow its activation to caspase-9 by fluorometric methods.

The cross-linking and light-scattering assays demonstrated that Cc binds to Apaf-1 independently of which ever tyrosine residue – Tyr46 or Tyr48 – is modified (Fig. 3). In fact, the light scattering of Apaf-1 increases upon addition of any of the Cc mutants (Fig. 3B).

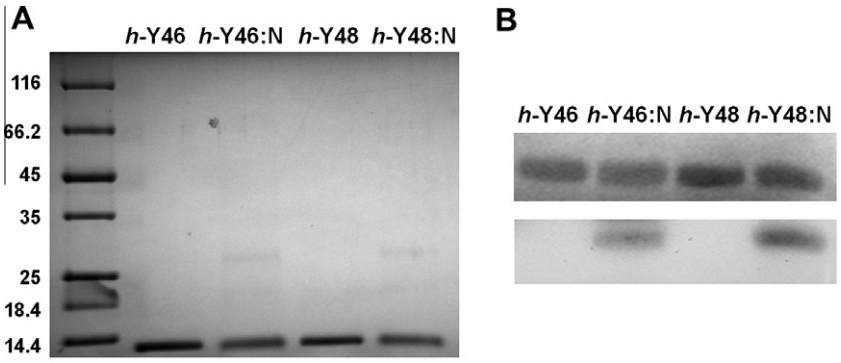

Fig.1. Determinationofpurityofnitrated C *c* samples.(A)SDS–PAGEelectrophoresisofnon-nitratedandnitrated *h*-Y46and *h*-Y48mutants.Eachprotein(1.3 μg)wasloaded ontoa12%SDS–PAGEgel.(B)Immunodetectionofnitrated C *c* mutantsbyWesternblotanalysisusinganti Tyr-NO 2 antibodies. *Upper* –Transferofnon-nitratedandnitrated *h*-Y46and *h*-Y48 samplestothenitrocellulosemembraneascorroboratedbyPonceauSsolutionstaining. *Lower* –Detectionof Tyr-NO 2 justinthe *h*-Y46:Nand *h*-Y48:N samplespreviouslysubmittedtothenitrationprotocol.

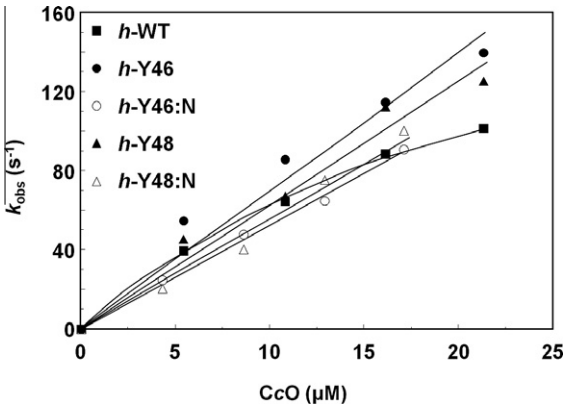

Fig.2. Dependenceof k_{obs} foroxidationof *h*-Y46and *h*-Y48,intheirnon-nitratedor nitratedforms,uponC *c*Oconcentration.Thereactionmixturecontained(inafinal volumeof0.8mL)10mMTris–HCl,pH6.5,2mMEDTA,0.07%dodecyl β-maltoside, 50mM KCl, 100 μM FMN, and 40 μM C*c*. All the experiments were performed at roomtemperature.Otherexperimentalconditionswereasdescribedunder Section 2.Theresultingkineticparametersaresummarizedin Table 1.

Table 1
KineticparametersforoxidationbyhorseC *c*Oofdifferenthuman C *c* speciesatpH6.5.

C*c* species	$k_2 \times 10^{-6}$ (M^{-1} s^{-1})	$K_A \times 10^{-4}$ (M^{-1})[a]	k'_{et} (s^{-1})[a]
h-WT	–	3.7	232
h-Y46	7.0	–	–
h-Y46:N	5.2	–	–
h-Y48	6.3	–	–
h-Y48:N	5.6	–	–

[a] K_A and k'_{et} valueswereestimatedasinRef.[20].

TheinabilityofthetwonitratedC *c* variantstoactivatecaspase-9 (Fig.4)suggeststhattheyyieldanon-functionalapoptosome.The tyrosine-by-phenylalaninemutationsthemselvesdonotalterthe abilityofC *c* toactivate caspase-9 at pH 7.5. Therefore, these two tyrosineresiduesarenotessentialfortriggeringapoptosisinvitro (Fig. 4). In contrast, nitration at positions 46 or 48 fully inhibits caspase-9 activation. At the highest C *c* concentration, only *h*-Y46:Nretains20%ofitsabilitytoactivatecaspase-9,aswasshown forthe *h*-Y74:Nspecies[21].Therefore,theinhibitoryeffectoncaspase-9 activation observed with the polynitrated *h*-WT species could be ascribed to the specific nitration of Tyr46 and/or Tyr48 rather than to any cooperative effect among different Tyr-NO 2 groups[20].

4. Discussion

Cc is involved in two opposite biological functions: cell life (mitochondrial respiration) and cell death (apoptosis), which are regulated by post-translational modifications such as nitration [20,34]. Actually, the addition of a –NO 2 group to any tyrosine residue yields different effects depending on the position of the modified residue at the heme protein structure.

In this context, it is worth noting that C *c* becomes a high-spin speciesuponTyr46orTyr48nitrationatphysiologicalpHalthough the overall folding remains unaltered [28], a finding that may explain the drop of ca. 100mV in the midpoint redox potential valueofthenitratedC *c* species[20].Suchadropinredoxpotential may roughly disrupt the cellular respiration. As inferred from the k_2 values for C *c*O reduction by non-nitrated and nitrated proteins at physiological pH, nitration at positions 46 and 48 barely affects theC *c* reactivity.However,C *c* nitratedatthesetwopositionsisno longerisopotentialwithcytochrome c_1 andmaythusbeunableto accept electrons from the cytochrome bc_1 complex. Actually, the excessinRNOSyieldedfromthefirstcomplexesoftherespiratory chainundernitroxidativestresscouldleadtoapositivenitration-drivenfeedbackcycle,withcytochromeb c_1 promotingtheincrease inRNOSandnitratedC *c*.

Upon leaving the mitochondria under nitroxidative stress, C *c* nitrated at positions 46 and 48 could inhibit the apoptosis signal by forming a non-functional apoptosome. However, the binding affinityofnitratedC *c* speciestowardsApaf-1issubstantiallylower than those of native C *c* (data not shown). Nitration of Tyr46 and Tyr48rearrangestheH-bondnetworkandturnsthealkalinetransitionintoaphysiologicallyrelevantprocess[32].Actually,thealkaline transition p K_a is shifted towards neutral pH values, with the concomitantreplacementofMet80byLys73orLys79ashemeaxial ligand.Asaconsequence,thealkalineformofnitratedC *c* ispredominantatpH7.5,whichistheoptimalpHvalueforcaspaseactivation. In the low-spin, alkaline structure of C *c*, the Ω-loop – which has previouslybeenreportedtobekeyfortheinteractionwithApaf-1 through residues Lys72 and/or Lys73 [20,35,36] – undergoes a substantialconformationalchange.Thisexplainstheassemblyofa non-functional apoptosome, which would be unable to activate caspasesandtodrivecellstoapoptosis.

Ithasrecentlybeenreportedthattyrosine48getsphosphorylatedunderhomeostaticconditions[37,38],withtheconcomitant effectontheavailabilityofC *c* toactivate caspases[39,40].Nitration and phosphorylation of C *c* at the same tyrosine residue are mutuallyexclusive[41]butinhibitC *c*-dependentcaspasesactivationwithasimilarefficiency.InthecaseofTyr48phosphorylation, the electron transfer is also inhibited [39]. In summary, Tyr48

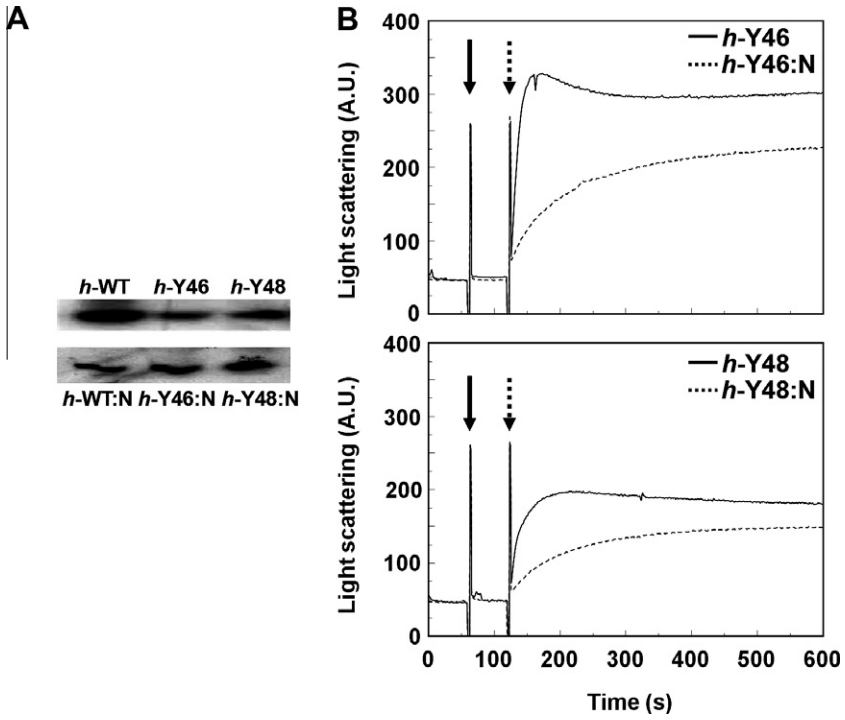

Fig. 3. Interaction between Apaf-1 and oxidized C *c* variants. (A) Cross-linking between Apaf-1 and the non-nitrated or nitrated *h*-Y46 and *h*-Y48 mutants, as detected by Western blot using antibodies against C *c*. (B) Formation of the complex between Apaf-1 and the non-nitrated (continuous line) or nitrated (dashed line) C *c* mutants, as determined by light scattering. The arrows stand for addition of Apaf-1 (solid line) and C *c* (dashed line) to the buffer solution.

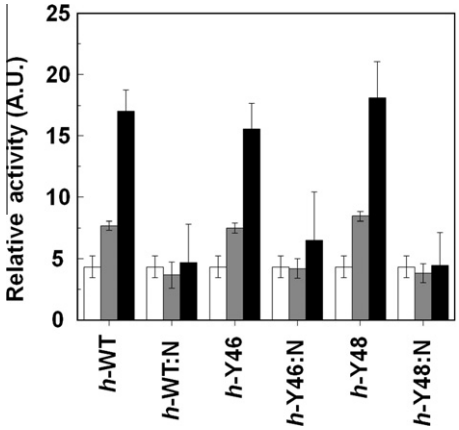

Fig. 4. C*c*-dependent activation of caspase-9 upon interaction of Apaf-1 with oxidized C *c* mutants. Caspase activation was determined by following the increase in fluorescence after substrate (Ac-LEHD-AFC) cleavage subsequent to incubation of Apaf-1 and PC9 with nitrated or non-nitrated C *c* mutant. C *c* concentrations were: 0 (white), 20 nM (grey) and 40 nM (black).

phosphorylation under homeostasis [37,38] and Tyr48 nitration under oxidative stress may act as anti-apoptotic switches that make C *c* fail in assembling a functional apoptosome.

Acknowledgements

The authors wish to thank the Spanish Ministry of Science and Innovation (BFU2009-07190) and the Andalusian Government (BIO198) for financial support.

References

[1] Bickers, D. and Athar, M. (2006) Oxidative stress in the pathogenesis of skin disease. J. Invest. Dermatol. 126, 2565–2575.

[2] Chen, Q., Vazquez, E.J., Moghaddas, S., Hoppel, C.K. and Lesnefsky, E.J. (2003) Production of reactive oxygen species by mitochondria. J. Biol. Chem. 278, 36027–36031.

[3] Szabó, C., Ischiropoulos, H. and Radi, R. (2007) Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat. Rev. Drug Discov. 6, 662–680.

[4] Radi, R., Cassina, A., Hodara, R., Quijano, C. and Castro, L. (2002) Peroxynitrite reactions and formation in mitochondria. Free Radic. Biol. Med. 33, 1451–1464.

[5] Pryor, W.A. and Squadrito, G.L. (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am. J. Physiol. Lung Cell. Mol. Physiol. 268, L699–L722.

[6] Denicola, A., Souza, J.M. and Radi, R. (1998) Diffusion of peroxynitrite across erythrocyte membranes. Proc. Natl. Acad. Sci. USA 95, 3566–3571.

[7] Jung, T., Bader, N. and Grune, T. (2007) Oxidized proteins: intracellular distribution and recognition by the proteasome. Arch. Biochem. Biophys. 462, 231–237.

[8] Su, J. and Groves, J.T. (2010) Mechanisms of peroxynitrite interactions with heme proteins. Inorg. Chem. 49, 6317–6329.

[9] Beckman, J.S., Ye, Y.Z., Anderson, P.G., Chen, J., Accavitti, M.A., Tarpey, M.M. and White, C.R. (1994) Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. Biol. Chem. Hoppe-Seyler 375, 81–88.

[10] Crow, J.P. and Beckman, J.S. (1995) Quantitation of protein tyrosine, 3-nitrotyrosine, and 3-aminotyrosine utilizing HPLC and intrinsic ultraviolet absorbance. Methods 7, 116–120.

[11] Ischiropoulos, H. (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch. Biochem. Biophys. 356, 1–11.

[12] Greenacre, S.A. and Ischiropoulos, H. (2001) Tyrosine nitration: localization, quantification, consequences for protein function and signal transduction. Free Radic. Res. 34, 541–581.

[13] Sacksteder, C.A., Qian, W.J., Knyushko, T.V., Wang, H., Chin, M.H., Lacan, G., Melega, W.P., Camp, D.G., Smith, R.D., Smith, D.J., Squier, T.C. and Bigelow, D.J. (2006) Endogenously nitrated proteins in mouse brain: links to neurodegenerative disease. Biochemistry 45, 8009–8022.

[14] Alonso, D., Encinas, J.M., Uttenthal, L.O., Boscá, L., Serrano, J., Fernández, A.P., Castro-Blanco, S., Santacana, M., Bentura, M.L., Richart, A., Fernández-Vizarra, P. and Rodrigo, J. (2002) Coexistence of translocated cytochrome *c* and nitrated protein in neurons of the rat cerebral cortex after oxygen and glucose deprivation. Neuroscience 111, 47–56.

[15] Cruthirs, D.L., Novak, L., Akhi, K.M., Sanders, P.W., Thompson, J.A. and MacMillan-Crow, L.A. (2003) Mitochondrial targets of oxidative stress during renal ischemia/reperfusion. Arch. Biochem. Biophys. 412, 27–33.

[16] Moore, G.R. and Pettigrew, G.W. (1990) Cytochromes *c*. Evolutionary, Structural, and Physicochemical Aspects, Springer-Verlag, New York.

[17] Kagan, V.E., Tyurin, V.A., Jiang, J., Tyurina, Y.Y., Ritov, V.B., Amoscato, A.A., Osipov, A.N., Belikova, N.A., Kapralov, A.A., Kini, V., Vlasova, I.I., Zhao, Q., Zou, M., Di, P., Svistunenko, D.A., Kurnikov, I.V. and Borisenko, G.G. (2005) Cytochrome *c* acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nat. Chem. Biol. 4, 223–232.
[18] Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 86, 147–157.
[19] Cai, J., Yang, J. and Jones, D.P. (1998) Mitochondrial control of apoptosis: the role of cytochrome *c*. Biochim. Biophys. Acta 1366, 139–149.
[20] Rodríguez-Roldán, V., García-Heredia, J.M., Navarro, J.A., De la Rosa, M.A. and Hervás, M. (2008) A comparative kinetic analysis of the reactivity of plant, horse, and human respiratory cytochrome *c* towards cytochrome *c* oxidase. Biochemistry 47, 12371–12379.
[21] García-Heredia, J.M., Díaz-Moreno, I., Nieto, P.M., Orzáez, M., Kocanis, S., Teixeira, M., Pérez-Payá, E., Díaz-Quintana, A. and De la Rosa, M.A. (2010) Nitration of tyrosine 74 prevents human cytochrome *c* to play a key role in apoptosis signaling by blocking caspase-9 activation. Biochim. Biophys. Acta Bioenerg. 1797, 981–993.
[22] Oursler, M.J., Bradley, E.W., Elfering, S.L. and Giulivi, C. (2005) Native, not nitrated, cytochrome *c* and mitochondria-derived peroxide drive osteoclast apoptosis. Am. J. Physiol. Cell. Physiol. 288, C156–C168.
[23] Batthyány, C., Souza, J.M., Durán, R., Cassina, A., Cerveñansky, C. and Radi, R. (2005) Time course and site(s) of cytochrome *c* tyrosine nitration by peroxynitrite. Biochemistry 44, 8038–8046.
[24] Cassina, A.M., Hodara, R., Souza, J.M., Thomson, L., Castro, L., Ischiropoulos, H., Freeman, B.A. and Radi, R. (2000) Cytochrome *c* nitration by peroxynitrite. J. Biol. Chem. 275, 21409–21415.
[25] Ueta, E., Kamatani, T., Yamamoto, T. and Osaki, T. (2003) Tyrosine-nitration of caspase 3 and cytochrome *c* does not suppress apoptosis induction in squamous cell carcinoma cells. Int. J. Cancer 103, 717–722.
[26] MacMillan-Crow, L.A., Cruthirds, D.L., Ahki, K.M., Sanders, P.W. and Thompson, J.A. (2001) Mitochondrial tyrosine nitration precedes chronic allograft nephropathy. Free Radic. Biol. Med. 31, 1603–1608.
[27] Jang, B. and Han, S. (2005) Biochemical properties of cytochrome *c* nitrated by peroxynitrite. Biochimie 88, 53–58.
[28] Díaz-Moreno, I., García-Heredia, J.M., Díaz-Quintana, A., Teixeira, M., and De la Rosa, M.A. (2011) Nitration of tyrosines 46 and 48 induces the specific degradation of cytochrome *c* upon change of the hemeiron state to high-spin. Biochim. Biophys. Acta Bioenerg. doi:10.1016/j.bbabio.2011.09.012.
[29] Appleby, C.A. (1969) Electron transport systems of *Rhizobium japonicum* : II. *Rhizobium* haemoglobin, cytochromes and oxidases in free-living (cultured) cells. Biochim. Biophys. Acta 172, 88–105.
[30] Zou, H., Li, Y., Liu, X. and Wang, X. (1999) An Apaf-1 cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. 274, 11549–11556.
[31] Malet, G., Martin, A.G., Orzáez, M., Vicent, M.J., Masip, I., Sanclimens, G., Ferrer-Montiel, A., Mingarro, I., Messeguer, A., Fearnhead, H.O. and Pérez-Payá, E. (2006) Small molecule inhibitors of Apaf-1-related caspase-3/-9 activation that control mitochondrial-dependent apoptosis. Cell Death Differ. 13, 1523–1532.
[32] Yoshikawa, S., Choc, M.G., O'Toole, M.C. and Caughey, W.S. (1977) An infrared study of CO binding to heart cytochrome *c* oxidase and hemoglobin A. J. Biol. Chem. 252, 5498–5508.
[33] Hervás, M., Navarro, J.A., Díaz-Quintana, A., Bottin, H. and De la Rosa, M.A. (1995) Laser-flash kinetic analysis of the fast-electron transfer from plastocyanin and cytochrome c_6 to photosystem-I. Experimental evidence on the evolution of the reaction mechanism. Biochemistry 34, 11321–11326.
[34] Nakagawa, H., Komai, N., Takusagawa, M., Miura, Y., Toda, T., Miyata, N., Ozawa, T. and Ikota, N. (2007) Nitration of specific tyrosine residues of cytochrome *c* is associated with caspase-cascade inactivation. Biol. Pharm. Bull. 30, 15–20.
[35] Kluck, R.M., Ellerby, L.M., Ellerby, H.M., Naiem, S., Yaffe, M.P., Margoliash, E., Bredesen, D., Mauk, A.G., Sherman, F. and Newmeyer, D.D. (2000) Determinants of cytochrome *c* pro-apoptotic activity. The role of lysine 72 trimethylation. J. Biol. Chem. 275, 16127–16133.
[36] Yu, T., Wang, X., Purring-Koch, C., Wei, Y. and McLendon, G.L. (2001) A mutational epitope for cytochrome *c* binding to the apoptosis protease activation factor-1. J. Biol. Chem. 276, 13034–13038.
[37] Yu, H., Lee, I., Salomon, A.R., Yu, K. and Hüttemann, M. (2008) Mammalian liver cytochrome *c* is tyrosine-48 phosphorylated in vivo, inhibiting mitochondrial respiration. Biochim. Biophys. Acta 1777, 1066–1071.
[38] Hüttemann, M., Pecina, P., Rainbolt, M., Sanderson, T.H., Kagan, V.E., Samavati, L., Doan, J.W. and Lee, I. (2011) The multiple functions of cytochrome *c* and their regulation in life and death decisions of the mammalian cell: from respiration to apoptosis. Mitochondrion 11, 369–381.
[39] Pecina, P., Borisenko, G.G., Belikova, N.A., Tyurina, Y., Pecinova, A., Lee, I., Samhan-Arias, A.K., Przyklenk, K., Kagan, V.E. and Huttemann, M. (2010) Phosphomimetic substitution of cytochrome *c* tyrosine 48 decreases respiration and binding to cardiolipin and abolishes ability to trigger downstream caspase activation. Biochemistry 49, 6705–6714.
[40] García-Heredia, J.M., Díaz-Quintana, A., Salzano, M., Orzáez, M., Pérez-Payá, E., Teixeira, M., De la Rosa, M.A. and Díaz-Moreno, I. (2011) Tyrosine phosphorylation turns alkaline transition into a biologically relevant process and makes human cytochrome *c* behave as an anti-apoptotic switch. J. Biol. Inorg. Chem. 16, 1155–1168.
[41] Reinehr, R., Görg, B., Höngen, A. and Häussinger, D. (2004) CD95-tyrosine nitration inhibits hyperosmotic and CD95 ligand-induced CD95 activation in rat hepatocytes. J. Biol. Chem. 279, 10364–10373.