ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 10, pp. 1128-1132. © Pleiades Publishing, Inc., 2006.
Published in Russian in Biokhimiya, 2006, Vol. 71, No. 10, pp. 1392-1398.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM06-112, September 10, 2006.

Regulation of Cytochrome *c* Peroxidase Activity by Nitric Oxide and Laser Irradiation

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Received April 19, 2006
Revision received May 26, 2006

Abstract—Apoptosis can be induced by activation of so-called "death receptors" (extrinsic pathway) or multiple apoptotic factors (intrinsic pathway), which leads to release of cytochrome *c* from mitochondria. This event is considered to be a point of no return in apoptosis. One of the most important events in the development of apoptosis is the enhancement of cytochrome *c* peroxidase activity upon its interaction with cardiolipin, which modifies the active center of cytochrome *c*. In the present work, we have investigated the effects of nitric oxide on the cytochrome *c* peroxidase activity when cytochrome *c* is bound to cardiolipin or sodium dodecyl sulfate. We have observed that cytochrome *c* peroxidase activity, distinctly increased due to the presence of anionic lipids, is completely suppressed by nitric oxide. At the same time, nitrosyl complexes of cytochrome *c*, produced in the interaction with nitric oxide, demonstrated sensitivity to laser irradiation (441 nm) and were photolyzed during irradiation. This decomposition led to partial restoration of cytochrome *c* peroxidase activity. Finally, we conclude that nitric oxide and laser irradiation may serve as effective instruments for regulating the peroxidase activity of cytochrome *c*, and, probably, apoptosis.

DOI: 10.1134/S0006297906100117

Key words: apoptosis, cytochrome *c*, cardiolipin, nitric oxide, laser irradiation, peroxidase activity

Programmed cell death (apoptosis) is one of the most interesting and important mechanisms that maintain cell and tissues during evolution. There are two pathways of apoptosis: extrinsic (receptor depending) and intrinsic (induced by specific proapoptotic stimuli) [1-4]. Both pathways activate caspase (serine proteinases) and lead to cell death [1, 2, 5-8]. Mitochondria play a crucial role in the intrinsic pathway of apoptosis by releasing cytochrome *c*, and this event is assumed to be a point of no return [1, 3, 9]. The release of cytochrome *c* is preceded by an increase in its peroxidase activity, as a result of interaction between cytochrome *c* and anionic lipids such as cardiolipin, phosphatidylserine, and sodium dodecyl sulfate (SDS) [10, 11]. It is assumed that peroxidative oxidation of membrane lipids and proteins can damage adenine nucleotide translocator (ANT) and contribute to the formation of permeability transition pore, swelling and disruption of mitochondria, and as a result the release of cytochrome *c* into the cytoplasm. On the other hand, cytochrome *c* can also be released from mitochondria through megapores in the outer mitochondrial membrane created due to peroxidase oxidation of membrane lipids [10, 11].

Nitric oxide can produce nitrosyl complexes in their interaction with heme-containing proteins [12]. In most cases, hemeprotein nitrosyl complexes (except guanylate cyclase) suppress their activity. Recently, it has been shown that complexes of cytochrome *c* with cardiolipin can form nitrosyl complexes in the reaction with nitric oxide, decreasing the peroxidase activity of cytochrome *c* [13]. The present paper describes cytochrome *c* nitrosyl complexes produced in the reaction of cytochrome *c* with cardiolipin or SDS and demonstrates their photosensitivity to laser radiation (He-Cd laser, 441 nm) [14]. Photolysis of these nitrosyl complexes can restore the cytochrome *c* peroxidase activity.

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MATERIALS AND METHODS

Reagents. Horse heart cytochrome *c* and SDS (Sigma, USA) and tetraoleoyl cardiolipin and palmitoyl stearoyl phosphatidylcholine (Avanti Polar Lipids, USA) were used in the experiments.

Preparation of liposomes. Cardiolipin–phosphatidylcholine liposomes (1 : 1 ratio) were prepared by mixing lipid chloroform solutions, removing chloroform by argon flow, subsequent addition of phosphate buffer (10 mM, pH 7.4) and intensive stirring. Finally, the sample was subjected to sonication in water bath for 15 min to produce a semi-transparent opalescent suspension. Total lipid concentration was 10 mM.

Nitric oxide solution. To prepare nitric oxide solution (100 ml) in 10 mM phosphate buffer (pH 7.4), oxygen was first removed by bubbling with argon for 15 min, and then the solution was bubbled with NO for 15 min to obtain saturated nitric oxide solution (~2 mM).

Luminol-enhanced chemiluminescence. Cytochrome *c* peroxidase activity was measured by means of luminol-enhanced chemiluminescence. LKB 1251 (LKB, Sweden) and KhLM-3 (BiKap, Russia) chemiluminometers were used in the experiments. Samples contained cytochrome *c* (10 μM), cardiolipin–phosphatidylcholine liposomes (20-500 μM) or SDS (0.5-10 mM), hydrogen peroxide (0.1 mM), and luminol (0.5 mM). All experiments were performed under constant stirring and at 22°C.

Measurement of nitric oxide concentration. Nitric oxide concentration was evaluated by a NO-sensitive electrode (World Precision Instruments, USA). All experiments were made in an air-impermeable chamber.

RESULTS

It is known that cytochrome *c* itself demonstrates a very low peroxidase activity, but in the presence of cardiolipin or SDS this activity can be dramatically increased [10, 15]. In the present work, we have studied cytochrome *c* peroxidase activity by means of luminol-enhanced chemiluminescence. This assay is based on peroxidative oxidation of luminol in the presence of hydrogen peroxide catalyzed by cytochrome *c*, which results in light emission. In Fig. 1 experimental data is presented that demonstrates the ability of cytochrome *c* to produce chemiluminescence in the presence of cardiolipin (in cardiolipin–phosphatidylcholine liposomes, 1 : 1 ratio) or SDS. It can be seen that cardiolipin or SDS together with cytochrome *c* demonstrate an increase in peroxidase activity compared to the peroxidase activity of cytochrome *c* alone. The cytochrome *c* peroxidase activity in the presence of cardiolipin was several-fold higher than that in the presence of SDS (Fig. 1, curves *2* and *3*).

It was interesting to know how cardiolipin/cytochrome *c* ratio can affect the peroxidase activity. Taking into consideration that the cardiolipin molecule is negatively charged (–2) and cytochrome *c* is positively charged (+8), we can expect that neutralization of this complex and its rearrangement occurs at the ratio 4 : 1. The first curve in Fig. 2 demonstrates the results of these experiments. It is obvious that up to the ratio of 4 : 1 the cardiolipin–cytochrome *c* complex peroxidase activity increases slowly. Increase in this ratio from 4 : 1 to 10 : 1 leads to rapid increase in peroxidase activity. Further increase in cardiolipin/cytochrome *c* ratio did not increase the peroxidase activity.

Curve *2* in Fig. 2 demonstrates the effect of SDS/cytochrome *c* ratio on chemiluminescence intensi-

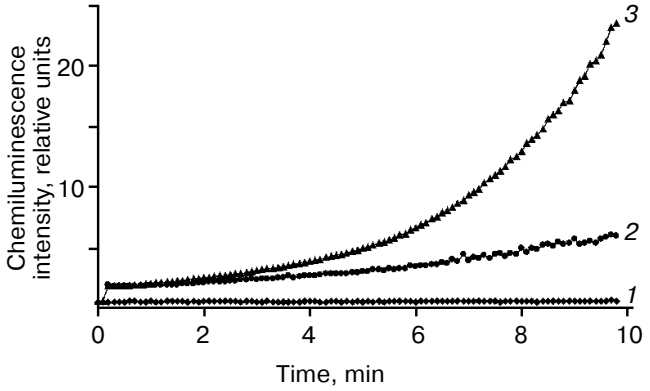

Fig. 1. Effects of cardiolipin and SDS on cytochrome *c* peroxidase activity measured by luminol-enhanced chemiluminescence. Sample content: *1*) cytochrome *c* (10 μM); *2*) cytochrome *c* (10 μM) + SDS (500 μM); *3*) cytochrome *c* (10 μM) + cardiolipin–phosphatidylcholine liposomes (1 : 1, 500 μM). All samples were prepared in 10 mM phosphate buffer (pH 7.4). H_2O_2 (100 μM) and luminol (500 μM) were added immediately before measurements.

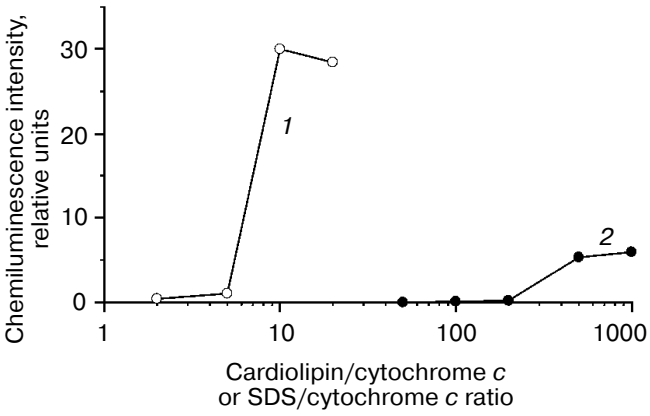

Fig. 2. Effects of cardiolipin/cytochrome *c* and SDS/cytochrome *c* ratio on cytochrome *c* peroxidase activity measured by luminol-enhanced chemiluminescence. Sample content: *1*) cytochrome *c* (10 μM) and cardiolipin (20-200 μM); *2*) cytochrome *c* (10 μM) and SDS (0.5-10 mM). All samples were prepared in 10 mM phosphate buffer (pH 7.4). H_2O_2 (100 μM) and luminol (500 μM) were added immediately before measurements.

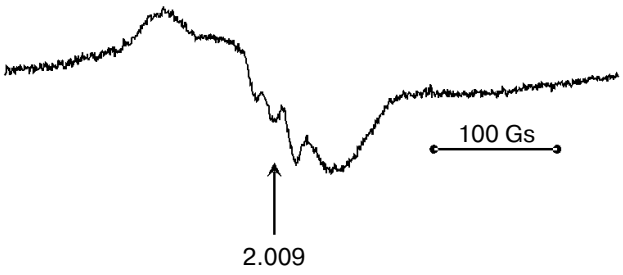

Fig. 3. ESR spectrum of cytochrome *c* nitrosyl complexes formed in the presence of cardiolipin. Sample content: cytochrome *c* (50 μM), cardiolipin–phosphatidylcholine liposomes (1 : 1, 0.5 mM), NO solution in phosphate buffer (0.2 mM). Sample volume 0.5 ml. ESR spectra were recorded at 77K. In the absence of cardiolipin, no ESR spectra were detected (data not shown). Registration conditions: magnetic field, 3300 Gs; scan range, 500 Gs; receiver gain, 5000; modulation amplitude, 1 Gs; microwave power, 10 mW; scan time, 4 min; time constant, 0.1 sec.

ty. It is seen that the SDS–cytochrome *c* complex does not show expressed peroxidase activity up to the ratio of 200 : 1. Subsequent increasing of this ratio to 500 : 1 leads to dramatic increase in chemiluminescence intensity (more than 35-fold higher) and peroxidase activity. Further increase in this ratio did not result in further increase in cytochrome *c* peroxidase activity.

The mechanism of cytochrome *c* peroxidase activity augmentation based on rearrangement in its spatial structure is described in the literature [16-18]. As a result of this rearrangement, the accessibility of hydrogen peroxide to cytochrome *c* heme iron is increased [16-18]. It has been shown by ESR assay that cytochrome *c* itself (in the absence of anionic lipids) can interact with nitric oxide [14]. Unfortunately, the kinetics of this reaction is slow and has a small yield. Recently, we demonstrated the acceleration of this interaction in the presence of cardiolipin [19]. In the present paper, we have studied the effects of nitric oxide on cytochrome *c*–cardiolipin complex by means of luminol-enhanced chemiluminescence.

To study the interaction of cytochrome *c* and nitric oxide in the presence of anionic lipids, we used a low-temperature ESR assay. Figure 3 shows the ESR spectrum of cytochrome *c* nitrosyl complexes formed in the presence of cardiolipin. This picture demonstrates the cytochrome *c* nitrosyl complex ESR signal produced at cardiolipin/cytochrome *c* ratio 10 : 1 [13, 16]. In the absence of cardiolipin the ESR signal was not detected (data not shown).

To confirm the cytochrome *c* NO-binding capacity in the presence of SDS, we used a NO-sensitive electrode. In Fig. 4, we present the results of these experiments. One can see that cytochrome *c* itself or SDS did not affect nitric oxide concentration in solution after addition of NO solution. In contrast, when both of them were present in the sample a decrease in NO concentration in the system could be seen. Thus, we conclude that SDS can facilitate the interaction between cytochrome *c* and nitric oxide. These findings confirm that the active center of cytochrome *c* in the presence of cardiolipin or SDS becomes more accessible not only to hydrogen peroxide, but also to nitric oxide. This reaction may lead to cytochrome *c* nitrosyl complex formation, which contributes to the rearrangement of cytochrome *c* spatial structure [13].

In the next series of experiments, we studied the effects of nitric oxide on cytochrome *c* peroxidase activity in the presence of anionic lipids by means of luminol-enhanced chemiluminescence. These results are presented in Fig. 5. One can see that addition of nitric oxide to cytochrome *c*–cardiolipin (Fig. 5, curve *3*) or cytochrome *c*–SDS complex (Fig. 5, curve *4*) leads to the complete disappearance of chemiluminescence (and consequently peroxidase activity). We suggest that this effect of nitric oxide is based on cytochrome *c* nitrosyl complex formation.

There are two questions to be answered: is cytochrome *c* nitrosyl complex stable, and is it possible to restore the cytochrome *c* peroxidase activity? Recently it has been shown that cytochrome *c* nitrosyl complexes (formed in the absence of anionic lipids) demonstrate photosensitivity to laser radiation and can be photolyzed into cytochrome *c* and nitric oxide [14, 16].

We have observed that cytochrome *c* nitrosyl complexes formed in the presence of cardiolipin or SDS demonstrate the photosensitivity and can dissociate upon laser irradiation.

The table demonstrates the effect of He-Cd laser irradiation on cytochrome *c* nitrosyl complexes formed in the presence of cardiolipin or SDS. Row 1 reveals a low peroxidase activity of cytochrome *c* itself. Addition of

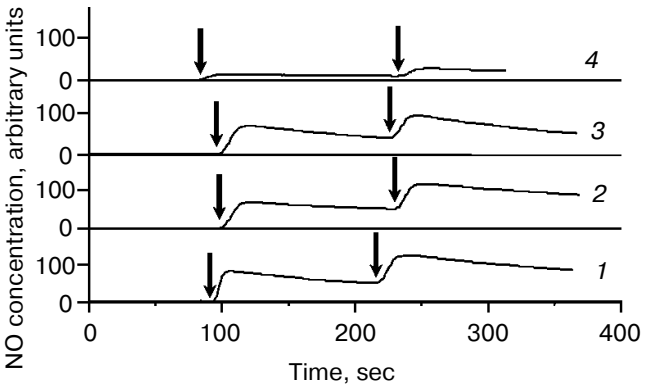

Fig. 4. Effect of SDS on NO binding to cytochrome *c* measured with a NO-sensitive electrode. Sample content: *1*) 10 mM phosphate buffer (pH 7.4) + NO (4 μM); *2*) cytochrome *c* (20 μM) + NO (4 μM); *3*) SDS (400 μM) + NO (4 μM); *4*) cytochrome *c* (20 μM) + SDS (400 μM) + NO (4 μM). Sample volume 1 ml. Arrows indicate the moments of NO (4 μM) addition.

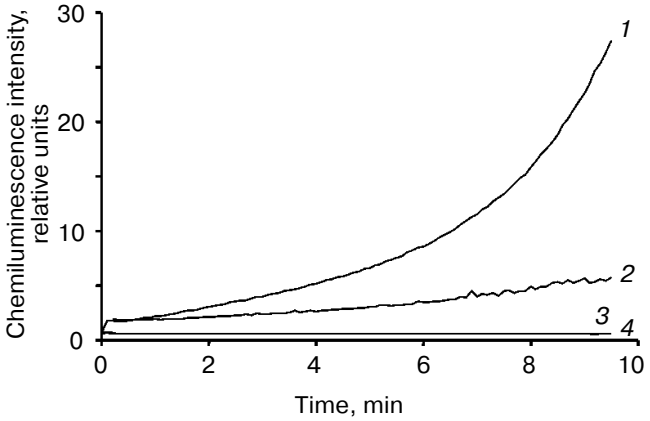

Fig. 5. Effect of nitric oxide on cytochrome *c* peroxidase activity in the presence of cardiolipin–phosphatidylcholine liposomes or SDS. Sample content: *1*) cytochrome *c* (10 μM) + cardiolipin–phosphatidylcholine liposomes (0.5 mM); *2*) cytochrome *c* (10 μM) + SDS (0.5 mM); *3*) cytochrome *c* (10 μM) + cardiolipin–phosphatidylcholine liposomes (0.5 mM) + NO (0.7 mM); *4*) cytochrome *c* (10 μM) + SDS (0.5 mM) + NO (0.7 mM). All samples were prepared in 10 mM phosphate buffer (pH 7.4). Incubation time 10 min. H_2O_2 (100 μM) and luminol (500 μM) were added immediately before measurement.

cardiolipin or SDS to cytochrome *c* (cardiolipin/cytochrome *c* ratio 20 : 1 or SDS/cytochrome *c* ratio 100 : 1) led to an increase in chemiluminescence intensity (and cytochrome *c* peroxidase activity) (row 2). The addition of nitric oxide decreased the cytochrome *c* peroxidase activity (row 3), apparently by the formation of cytochrome *c* nitrosyl complexes. Laser irradiation photolyzed the cytochrome *c* nitrosyl complexes and partly restored cytochrome *c* peroxidase activity (row 4). The data in the table demonstrates that laser irradiation induces photolysis of cytochrome *c* nitrosyl complexes and thus increases the cytochrome *c* peroxidase activity detected by means of luminol-enhanced chemiluminescence.

DISCUSSION

In the mitochondrial respiratory chain, cytochrome *c* can act not only as an electron carrier but is able to demonstrate enhanced peroxidase activity upon interaction with anionic lipids (such as cardiolipin, phosphatidylserine, or SDS) [10, 17-19]. Heme iron in the active site of cytochrome *c* has six coordination bonds. Four of them belong to the porphyrin ring and the fifth and the sixth bonds – to His18 and Met80, respectively. This means that access to heme iron is hindered even to small ligands such as hydrogen peroxide or nitric oxide. The presence of anionic lipids affects the structure of the active center of cytochrome *c* and increases its accessibility for hydrogen peroxide and nitric oxide [20, 21]. This phenomenon is the result of deformation or breakage of the iron–Met80 bond [19, 22].

High peroxidase activity of cytochrome *c* bound to cardiolipin of the mitochondrial membrane induces oxidation of membrane proteins and lipids, and as a result increasing of mitochondrial permeability for cytochrome *c* and its release into the cytoplasm. There are two possible mechanisms of cytochrome *c* release from mitochondria, and both of them are based on the enhancement of cytochrome *c* peroxidase activity. First is the oxidation of membrane lipids by the cytochrome *c*-mediated peroxidase reaction and formation of megapores in the outer mitochondrial membrane for cytochrome *c* release [23]. Second, cytochrome *c* can damage the ANT (adenine nucleotide translocator) of the inner mitochondrial membrane and form a membrane channel for cytochrome *c* release [24]. Mitochondrial matrix swelling, damage to the outer mitochondrial membrane, and cytochrome *c* release into the cytoplasm are consequences of this process [25-27].

Is it possible to control the cytochrome *c* peroxidase activity? It is well known that interaction of nitric oxide and hemeproteins leads to the suppression of their enzymatic activity [28]. Recently, we have demonstrated that

Effects of NO and laser irradiation on cytochrome *c* chemiluminescence in the presence of cardiolipin (CL) or SDS

Sample content	Chemiluminescence intensity	Sample content	Chemiluminescence intensity
Cytochrome *c*	100.00 ± 28.47		
+CL	246.06 ± 41.45	+SDS	482.64 ± 45.60
+CL + NO	45.44 ± 20.33	+SDS + NO	105.28 ± 27.54
+CL + NO + irradiation	159.68 ± 16.89	+SDS + NO + irradiation	256.95 ± 70.60

Note: Sample content: cytochrome *c* (10 μM); cytochrome *c* (10 μM) + cardiolipin–phosphatidylcholine liposomes (1 : 1, 0.2 mM) or cytochrome *c* (10 μM) + SDS (1 mM); cytochrome *c* (10 μM) + cardiolipin–phosphatidylcholine liposomes (0.2 mM) + NO (0.1 mM) or cytochrome *c* (10 μM) + SDS (1 mM) + NO (0.1 mM); cytochrome *c* (10 μM) + cardiolipin–phosphatidylcholine liposomes (0.2 mM) + NO (0.1 mM) + He-Cd laser irradiation (20 mW, 15 min) or cytochrome *c* (10 μM) + SDS (1 mM) + NO (0.1 mM) + He-Cd laser irradiation (20 mW, 15 min). All samples were prepared in 10 mM phosphate buffer (pH 7.4). Incubation time was 10 min. H_2O_2 (100 μM) and luminol (500 μM) were added immediately before measurements.

addition of nitric oxide to cytochrome *c* (incubated with cardiolipin or SDS) decreases the cytochrome *c* peroxidase activity as seen by chemiluminescence assay [29]. In other words, cytochrome *c* can form nitrosyl complexes inhibiting the interaction of cytochrome *c* with hydrogen peroxide. We suppose that if cytochrome *c* peroxidase activity is a prerequisite of apoptosis, then suppression of this activity by nitric oxide can be used to control apoptosis. In this case, NO can serve as an antiapoptotic agent.

We recently observed that cytochrome *c* nitrosyl complexes are photosensitive [29]. Irradiation of cytochrome *c* nitrosyl complexes with He-Cd laser (441 nm) can cause the photolytic decomposition of this complexes and increase in cytochrome *c* peroxidase activity. This fact means that, if similar events can happen in cells, then laser irradiation can serve as a proapoptotic factor.

Finally, we conclude that cytochrome *c* can form complexes with cardiolipin that possess enhanced peroxidase activity [17]. These complexes can be involved in the damage to the ANT or can oxidize the mitochondrial membrane and in this way lead to the formation of the permeability transition pore and cytochrome *c* release into the cytoplasm. Thus, our studies show that nitric oxide and laser irradiation can serve as instruments to control the peroxidase activity of cytochrome *c* and, perhaps, apoptosis.

This study was supported by the Russian Foundation for Basic Research (grants 06-04-49296 and 05-04-49765).

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