

Dihydroquercetin (Taxifolin) and Other Flavonoids as Inhibitors of Free Radical Formation at Key Stages of Apoptosis

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Abstract—Formation of free radicals in mitochondria plays a key role in the development of apoptosis, which includes formation of superoxide by the respiratory chain, formation of radicals by cytochrome *c*–cardiolipin complex in the presence of hydrogen peroxide or lipids, and chain lipid peroxidation resulting in cytochrome *c* release from mitochondria and initiation of the apoptotic cascade. In this work the effect of taxifolin (dihydroquercetin) and some other antioxidants on these three radical-producing reactions was studied. Peroxidase activity of the complex of cytochrome *c* with dioleoyl cardiolipin estimated by chemiluminescence with luminol decreased by 50% with quercetin, taxifolin, rutin, Trolox, and ionol at concentrations 0.7, 0.7, 0.8, 3, and 10 μ M, respectively. The lipid radical production detected by coumarin C-525-activated chemiluminescence decreased under the action of rutin and taxifolin in a dose-dependent manner, so that a 50% inhibition of chemiluminescence was observed at the antioxidant concentrations of 3.7 and 10 μ M, respectively. Thus, these two radical-producing reactions responsible for apoptosis onset are inhibited by antioxidants at rather low concentrations. Experiments performed on liver slices and mash showed that taxifolin, quercetin, naringenin, and Trolox have low inhibitory effect on the lucigenin-dependent chemiluminescence in the tissue only at concentrations higher than 100 μ M.

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A large pool of data has accumulated suggesting for development of apoptosis a stage of free radical production [1-6], namely, the stage of lipid peroxidation [7, 8]. In particular, Nomura et al. found that overexpression of phospholipid glutathione peroxidase decelerates the development of apoptosis in cell culture, whereas knock-out of this enzyme leads to enhanced development of apoptosis [7, 9]. Note that the only proven function of this enzyme is suppression of lipid peroxidation. V. E. Kagan and coworkers [10] have shown that the oxidized

cardiolipin formation occurs already 4 h after inoculation of an apoptogen into the cell culture, whereas other apoptosis-associated events, such as cytochrome *c* release out of mitochondria, activation of caspases, and externalization of phosphatidylserine are developing for several additional hours. Cardiolipin was not oxidized and apoptosis did not develop in cytochrome *c*-deficient cells.

Apoptosis of functionally important cells leads to complications in degenerative disorders [11-13], so it should be minimized for prophylactics of these complications. At the same time, tumor cells have mechanisms defending them from apoptosis [14-16], and its stimulation underlies the effect of major anti-neoplastic drugs [15, 17-19]. So the problem of control over apoptosis using antioxidants is extremely important.

There are at least three sequential events in the development of apoptosis, in which free radicals seem to play a key role:

Abbreviations: BCL, bovine cardiolipin; CL, chemiluminescence; coumarin C-525, 2,3,5,6-1H,4H-tetrahydro-9-(2'-benzimidazolyl)quinolizino-(9,9a,1-gh)coumarin; DHQ, dihydroquercetin (taxifolin); NO, nitric oxide; SOD, superoxide dismutase; TOCL, 1,1',2,2'-tetraoleyl cardiolipin (sodium salt).

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– formation of superoxide radicals in the mitochondrial respiratory chain [20] with their subsequent conversion to hydrogen peroxide by mitochondrial superoxide dismutase (SOD);

– formation of secondary radicals [21] due to peroxidase and lipoxygenase activities of the cytochrome *c*–cardiolipin complex on the inner mitochondrial membrane [22, 23];

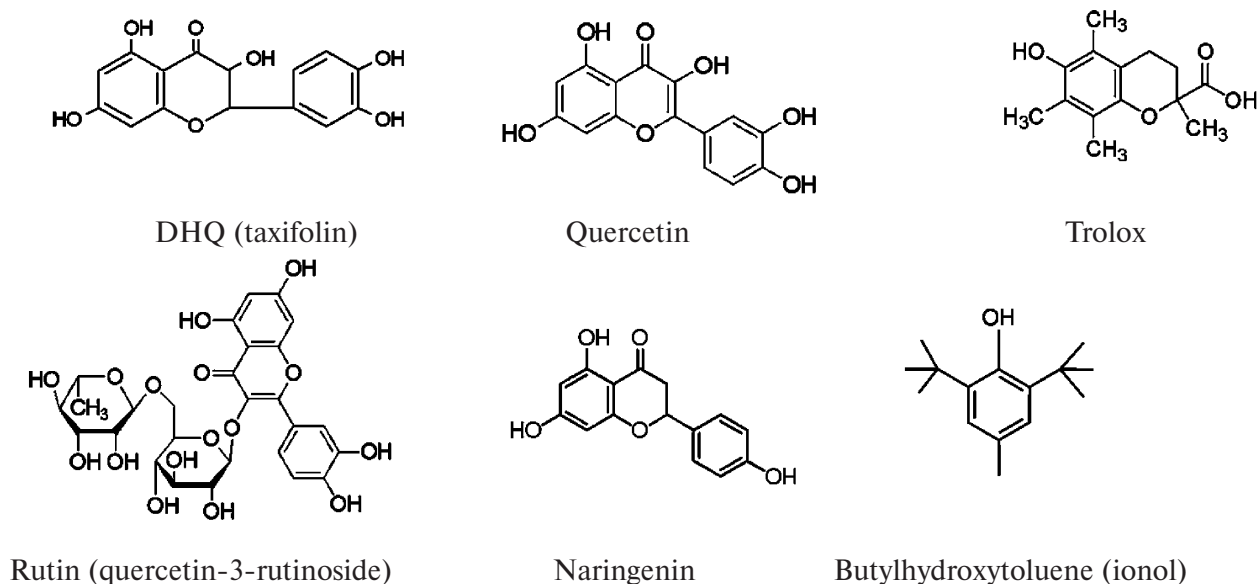
– chain lipid oxidation initiated by these secondary radicals, which leads to matrix swelling, cytochrome *c* release from mitochondria, and initiation of the apoptotic cascade [24–28].

In this work we have studied the effect of some antioxidants on these three stages of free radical formation. Superoxide radical production in tissue pieces and mash was determined from chemiluminescence (CL) in the presence of lucigenin [29], peroxidase activity of cytochrome *c*–cardiolipin complexes was estimated by means of CL in the presence of luminol, and lipid radical production initiated by the cytochrome *c*–cardiolipin complex was measured by the method of quinoxalino-coumarin C-525-activated CL. It is shown that the studied antioxidants inhibit radical production in the peroxidase reaction with luminol at micromolar concentrations, in lipid radical production at one order higher concentration, and CL of tissue pieces ascribed to superoxide radical production in mitochondria only decreased by 30–50% at antioxidant concentration > 100 μ M. Thus, it has been demonstrated in model experiments that dihydroquercetin and other antioxidants, such as flavonoids, can inhibit development of apoptosis in its three points associated with free radical production.

MATERIALS AND METHODS

Chemicals. Cytochrome *c* from horse heart (99%) was purchased from Sigma (USA, Cat. No. C7752). Concentration was determined spectrophotometrically after reduction by ascorbic acid (99%; Aldrich, Germany) using extinction coefficient $\epsilon_{550} = 29,400$ liter/mol per cm [30]. Cardiolipin from bovine heart (BCL) (98% TLC; Sigma) or 1,1',2,2'-tetraoleyl cardiolipin (TOCL, disodium salt) were also used. Luminol (ICN Biomedicals, USA, Cat. No. 195038), coumarin C-525 (2,3,5,6-1H,4H-tetrahydro-9-(2'-benzoimidazolyl)-quinolizino-(9,9a,1-gh)coumarin) (NIOPIK, Moscow, Russia), and lucigenin (Sigma-Aldrich, USA) were used as CL activators. Hydrogen peroxide working solution was prepared from its 30% aqueous solution (Sigma-Aldrich). Its concentration was determined spectrophotometrically ($\epsilon_{230} = 72.1$ liter/mol per cm [16]). Phosphate buffer, pH 7.4, was prepared using $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (analytical grade; Khimmed, Russia). The following antioxidants were used: quercetin, rutin, naringenin, 2,6-di-*tert*-butyl-*p*-cresol (butylhydroxytoluene, ionol) (Sigma-Aldrich), Trolox (Fluka, Germany), dihydroquercetin (DHQ, taxifolin) (Khimdrevsesina, Russia). The chemical structures of the antioxidants used are shown on the Scheme.

Peroxidation catalyzed by cytochrome *c*–cardiolipin complex. Preparation of complexes between cytochrome *c* and tetraoleyl cardiolipin and measurement of peroxidase activity of these complexes was carried out as described in [31] with modifications. Aqueous solution of BCL was added to a solution of cytochrome *c* in phosphate buffer (final concentration 20 μ M) so that the BCL/cytochrome *c* ratio was 32 : 1. Following agitation at



Structures of antioxidants used

room temperature for 10 min, luminol and hydrogen peroxide were added (final concentrations 200 and 20 μM , respectively). Luminescence is due to oxidation of luminol by hydrogen peroxide catalyzed by the cytochrome *c*-TOCL complex. CL was recorded using a SmartLum 5773 chemiluminometer (Interoptica-C, Russia).

Solutions of antioxidants in methanol were placed into a cell directly before H_2O_2 addition and CL measuring; the final alcohol concentration was less than 10%. Control measurements of tryptophan fluorescence, cytochrome *c* absorption at 695 nm, and peroxidase activity of BCL-cytochrome *c* complex have shown that methanol taken at this concentration has no effect on any used parameters of activity of cytochrome *c* and its complexes. A series of kinetic curves was obtained at varied antioxidant concentrations. Antioxidant activity of the substance was estimated from decrease in CL intensity at the maximum of the curve.

Oxidation of natural cardiolipin catalyzed by its complex with cytochrome *c*. Effect of antioxidants on lipid radical production was studied in a system cytochrome *c* + BCL + coumarin C-525 + antioxidant. Under these conditions the cytochrome *c*-BCL complex possesses peroxidase activity, where BCL is an oxidized substrate and coumarin C-525 is a selective physical activator (sensitizer) of CL associated with lipid peroxidation. Cytochrome *c* (final concentration 20 μM), coumarin C-525 (final concentration 20 μM), and methanol solution of antioxidant were added to BCL in phosphate buffer (BCL/cytochrome *c* = 32 : 1). CL was recorded, and the plot of slow flash amplitude against the final antioxidant concentration was made.

Chemiluminescence of liver pieces. Experiments were carried out as described in [29]. Liver tissue pieces of 250 ± 10 mg wet weight in Hanks' solution (3125 μl) were placed into a cell of a CLM-3 chemiluminometer (Bikap,

Russia). Then lucigenin was added to the final concentration of 0.1 mM, and increase in CL was recorded. CL intensity was taken as the index of superoxide radical production rate in the tissue.

Effect of antioxidants on lucigenin-enhanced CL of liver pieces was determined in two ways. In the first case, antioxidant was added in the course of experiment, when the CL signal reached a plateau. In the second case, the liver pieces in the chemiluminometer cell were preincubated with the antioxidant for 5 min at 37°C, and luminescence in the presence of lucigenin was recorded as described in [29]. Antioxidants were dissolved in ethanol. The final concentration of ethanol was 950 μM , and the concentration of all other antioxidants was 157 μM . Samples containing the same amounts of ethanol without antioxidant served as controls. It has been shown that ethanol taken at used concentrations (<10%) has no effect on the CL of the tissue.

RESULTS

Inhibition of peroxidation catalyzed by cytochrome *c*-TOCL complex. Effect of antioxidants on the peroxidase activity of cytochrome *c* bound in a complex with TOCL was estimated from the luminol CL. Typical CL curves of luminol oxidation in the presence of varied amounts of DHQ and quercetin are shown in Fig. 1. One can see that CL intensity decreases and its development is delayed in the presence of antioxidant. Antioxidative effect can be quantitatively measured by CL amplitude (luminescence intensity at the maximum).

Figure 2 shows dose-dependent effects of four antioxidants: quercetin, dihydroquercetin (taxifolin), Trolox, and rutin. Activity of an antioxidant can be estimated by its concentration resulting in twofold decrease

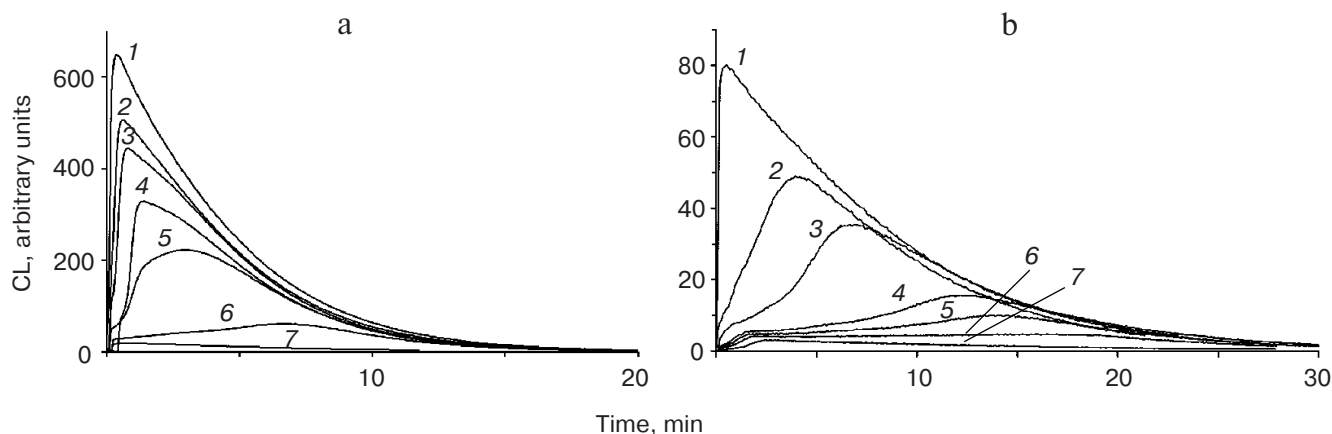


Fig. 1. Inhibition of peroxidase activity of cytochrome *c*-TOCL complex by dihydroquercetin (a) and quercetin (b). Concentration of cytochrome *c* was 7.5 μM , BCL – 240 μM , luminol – 50 μM , H_2O_2 – 50 μM , DHQ – 0, 0.25, 0.5, 0.66, 1.25, 2, and 4 μM (1-7, respectively), and quercetin – 0, 0.4, 0.8, 1.6, 2, 2.5, and 4 μM (1-7, respectively).

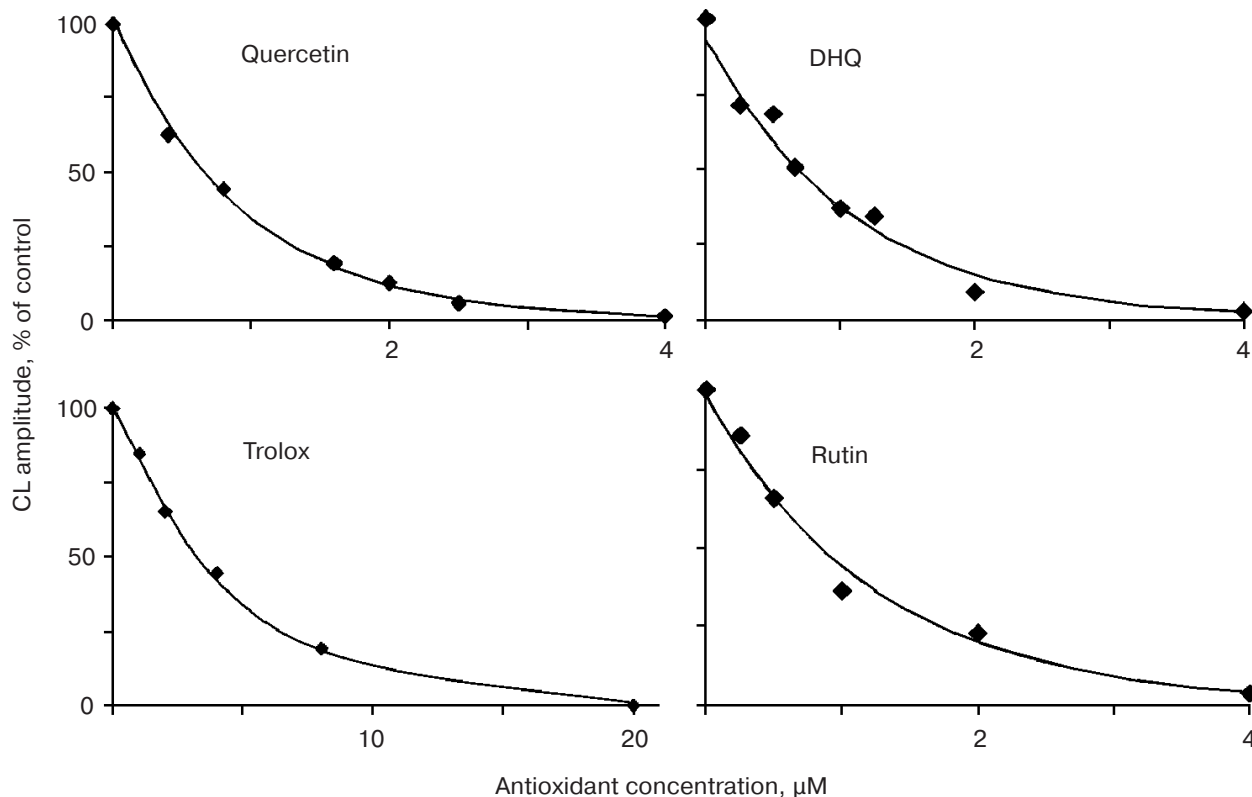


Fig. 2. Dose-dependent effects of antioxidants on peroxidase activity of cytochrome *c*-TOCL complex. CL amplitudes (% of control, that is, in the absence of antioxidant) are plotted on the ordinate. Experimental conditions are the same as in the legend to Fig. 1.

in CL amplitude. These concentrations are 0.7, 0.7, 3.7, and 0.8 μM , respectively. Thus, peroxidase activity of the cytochrome *c*-TOCL complex can be inhibited by the antioxidants taken at concentrations of about 1 μM .

Inhibition of radical production during oxidation of BCL complexed with cytochrome *c*. The cytochrome *c*-cardiolipin complex is known to catalyze lipid peroxidation [10, 23]. In this process, both hydrogen peroxide and lipid hydroperoxides, which are commonly present in a lipid system containing polyunsaturated fatty acids, appear to serve as substrates. One method allowing estimation of lipid hydroperoxide production is measurement of CL in the presence of activators such as quinoxalino-coumarins, in particular, coumarin C-525 [32, 33]. We found that coumarin C-525-activated CL in the cytochrome *c* + BCL system. Typical curves of such CL are shown in Fig. 3b. These curves are of complicated character and, in some cases, are composed of fast and slow components. In this work, we studied effects of antioxidants on the slow component, which is apparently dependent not only on peroxidation itself catalyzed by cytochrome *c*, but also by following development of chain oxidation due to initiation of chains by radicals produced in the peroxidation process.

Figure 3a (inset) shows that addition of DHQ leads to decrease in the CL maximum and elongation of the

latent period of its development. As in the above experiments, the CL amplitude was regarded as the index of antioxidant activity. Dependence of the amplitude on the DHQ concentration is shown in Fig. 3a. Similar results were obtained when other antioxidants, including rutin, were tested (Fig. 3b). It is noticeable that concentrations of DHQ and rutin at which 50% inhibition occurs (9 and 3.7 μM , respectively) are several times higher than those necessary for inhibition of peroxidase activity itself (compare with Fig. 2).

Do antioxidants influence primary production of superoxide radicals in living tissue? We judged superoxide radical production in liver tissue by luminescence of the tissue on injection of lucigenin into the ambient solution (Fig. 4, control). Luminescence developed for about 20 min after addition of lucigenin to a liver piece incubated at 37°C in the chemiluminometer cell followed by slow CL decrease. As shown earlier, this luminescence is oxygen-dependent and can be attributed to a reaction of lucigenin cation-radical with superoxide [29]. Unfortunately, the use of SOD is little demonstrative in this case because this enzyme hardly penetrates into the tissue.

Antioxidants were added to isolated liver pieces placed directly into a chemiluminometer cell and incubated for 5 min with following lucigenin addition and luminescence measurement. It was shown that antioxidant

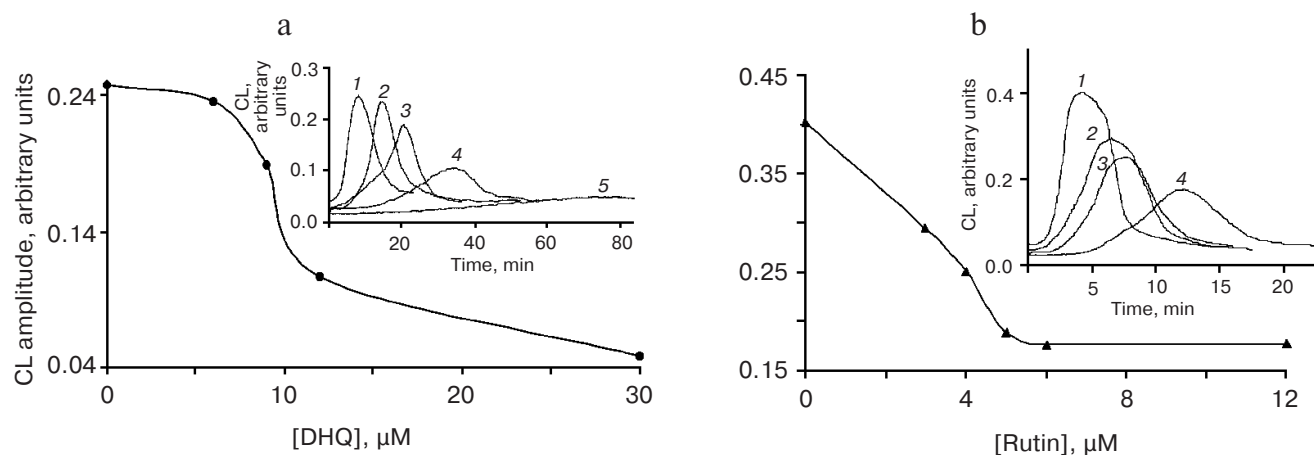


Fig. 3. Inhibition of lipid radical production on incubation of BCL with cytochrome *c* by DHQ (a) and rutin (b). The steady-state concentration of lipid dioxide radicals was estimated from CL intensity in the presence of quinolizino-coumarin C-525. The major plots show dependences of amplitude (CL intensity at the maximum) on the final concentrations of DHQ (a) and rutin (b) in samples. The kinetics of CL is shown in the inset; the signal in absence of coumarin was close to dark current (not shown). Measurement was begun right after addition of cytochrome *c* to phosphate buffer containing BCL, alcoholic solution of antioxidant, and C-525. Concentration of cytochrome *c* was 20 μM, molar ratio lipid/protein = 32 : 1, concentration of coumarin – 0, 6, 9, 12, and 30 μM (1-5, respectively), rutin – 0, 3, 4, and 6 μM (1-4, respectively).

became distributed between the solution and surface tissue layer, in which detected luminescence appeared, for 5 min of incubation. The CL curves in the presence of antioxidants in all cases were characterized by lower intensity than the control (Fig. 4, dashed line). Quercetin proved to be the most effective antioxidant, whereas DHQ, Trolox, ionol, and naringenin possessed lower activity.

We found that antioxidant solution added to the liver piece, while CL is recorded, also leads to decrease in luminescence. In another series of experiments, the effect of antioxidants was examined on a suspension of small liver pieces (~1 mm in diameter); effective concentrations of antioxidants and degree of CL inhibition were approximately the same as that for the larger tissue piece (data not shown).

It is notable that concentrations of antioxidants at which inhibition of lucigenin-dependent CL was observed was more than two orders higher than those leading to inhibition of luminol radical and lipid radical production by the cytochrome *c*–cardiolipin complexes. For instance, concentrations of DHQ and rutin leading to 50% decrease in luminol radical production in the peroxidation reaction were 0.8 and 0.7 μM, respectively, whereas the concentrations leading to the same inhibition of lipid radical production upon oxidation of BCL in its complex with cytochrome *c* were 9 and 3.7 μM, respectively. At the same time, inhibition of superoxide radical production in liver slices and small pieces was 30-40% at concentration of rutin and DHQ of 157 μM. Similar proportions are characteristic of the other antioxidants (compare Figs. 2 and 4).

Thus, biologically significant concentrations of antioxidants (<100 μM) have no considerable effect on

production of primary (superoxide) radicals in cells, but inhibit production of secondary radicals of lipids and other organic substances.

DISCUSSION

The sequence of events involved in the development of apoptosis is rather complex and seems to vary depending on the apoptotic agent and cell type. However, many facts suggest radical formation in mitochondria, which is accompanied by peroxidation of the inner membrane lipids, as the turning-point from life to programmed death. Apparently, all begins with a massive production of superoxide radicals by the respiratory chain, which is somehow associated with destabilization of its optimal activity. Superoxide is potentially hazardous for three reasons. First, its interaction with NO produced by mitochondrial NO-synthase leads to formation of highly toxic peroxynitrite possessing apoptogenic activity [34-36]. Second, due to reduction of Fe³⁺ to Fe²⁺, superoxide can extract iron ions from cellular depots and, possibly, from the respiratory chain, thus enhancing the danger of lipid peroxidation [37-39]. Finally, hydrogen peroxide formed from superoxide by mitochondrial SOD, serves as a substrate for peroxidases including the cytochrome *c*–cardiolipin complex [10, 23, 31, 40]. Lipids can serve as the second substrate, and lipid peroxidation damages mitochondria and leads to apoptosis. Thus, three sequential events: primary (superoxide) radical production by the mitochondrial respiratory chain, formation of lipid radicals by the cytochrome *c*–cardiolipin complex, and chain oxidation of lipids initiated by these radicals can be thought as

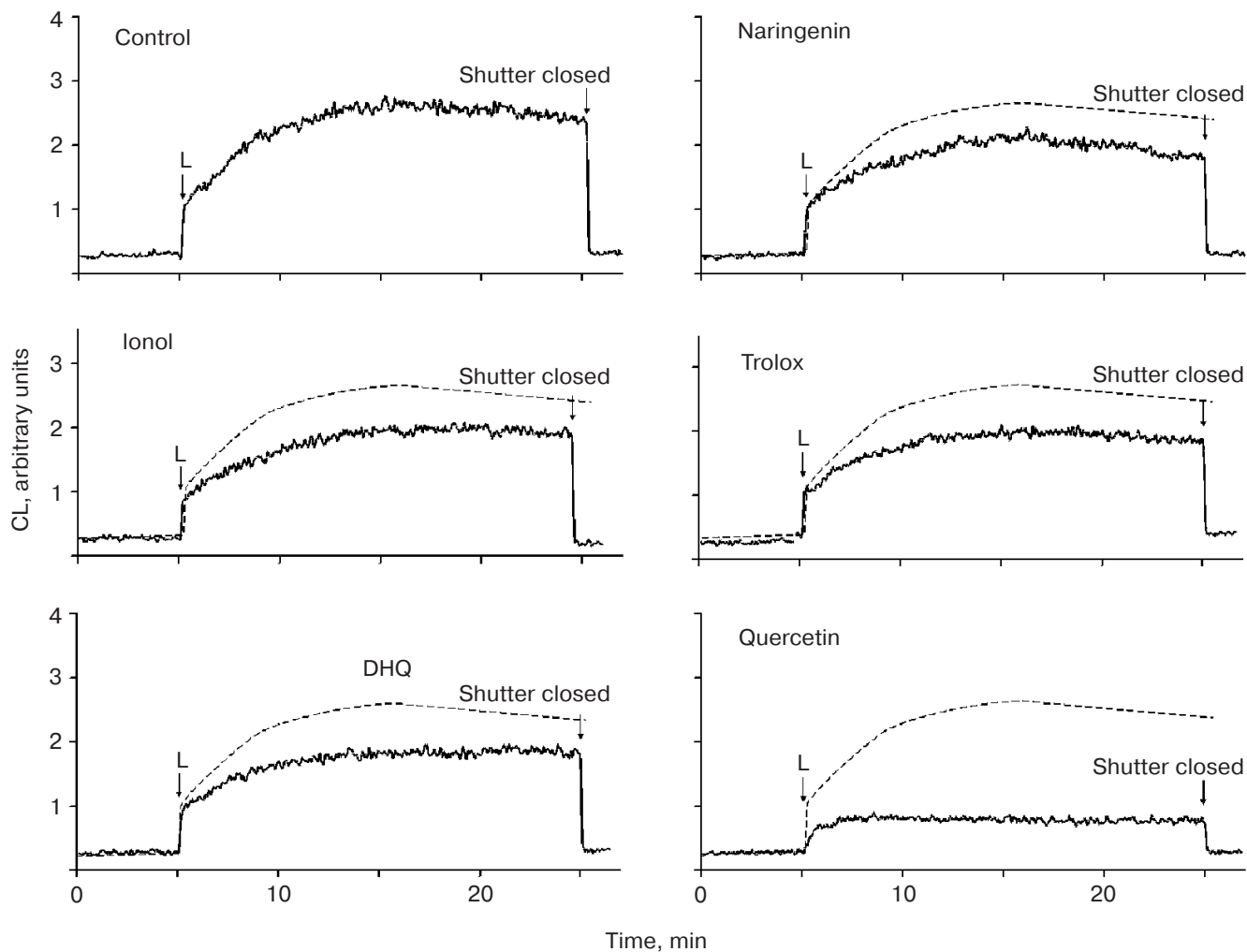


Fig. 4. Effect of preincubation with antioxidants on development of lucigenin-dependent CL of rat liver tissue piece. Liver pieces were incubated with antioxidant for 5 min at 37°C immediately before measurements. Incubation medium: 0.1 mM of lucigenin (L) in 3125 μ l of Hanks' solution, pH 7.4. The volumes of antioxidant solutions were 30–50 μ l. The final concentration of ionol was 950 μ M, other antioxidants – 157 μ M.

keys for the development of apoptosis. Control over radical production at these three stages could exert control over apoptosis.

In this work, we have shown that dihydroquercetin and other antioxidants decelerate radical production at all three enumerated stages, but to widely different degree: effective concentration of antioxidants in regards to CL of tissue in the presence of lucigenin was two orders of magnitude higher than that inhibiting peroxidase activity of cardiolipin complexes (luminol-dependent CL) and one order higher than that inhibiting lipid radical production by these complexes (C-525-activated CL). It is obvious that antioxidants influence all three radical-producing reactions, which are crucial for the development of apoptosis, but the most significant is their effect on peroxidase activity of the cytochrome *c*–cardiolipin complex.

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