ATP Induces Conformational Changes in Mitochondrial Cytochrome c Oxidase

EFFECT ON THE CYTOCHROME c BINDING SITE

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ATP influences the kinetics of electron transfer from cytochrome c to mitochondrial oxidase both in the membrane-embedded and detergent-solubilized forms of the enzyme. The most relevant effect is on the so-called “high affinity” binding site for cytochrome c which can be converted to “low affinity” by millimolar concentrations of ATP (Ferguson-Miller, S., Brautigan, D. L., and Margolish, E. (1976) J. Biol. Chem. 251, 1104-1115). This phenomenon is characterized at the molecular level by the following features. (i) ATP triggers a conformational change on the water-exposed surface of cytochrome c oxidase; (ii) in this process, carboxyl groups forming the cluster of negative charges responsible for binding cytochrome c change their accessibility to water-soluble protein modifier reagents; (iii) as a consequence the electrostatic field that controls the enzyme-substrate interaction is altered and cytochrome c appears to bind differently to oxidase; (iv) photolabeling experiments with the enzyme from bovine heart and other eukaryotic sources show that ATP cross-links specifically to the cytoplasmic subunits IV and VIII. Taken together, these data indicate that ATP can, at physiological concentration, bind to cytochrome c oxidase and induce an allosteric conformational change, thus affecting the interaction of the enzyme with cytochrome c. These findings raise the possibility that the oxidase activity may be influenced by the cell environment via cytoplasmic subunit-mediated interactions.

Cytochrome c oxidase, the terminal enzyme of the respiratory chain of most aerobic organisms, is a membrane protein complex containing two hemes a, two copper atoms, and a number of subunits varying with the degree of evolution of the species (1, 2). In eukaryotes the three largest polypeptides are encoded by mitochondrial genes and they are responsible for the electron transfer and proton pumping activities of the enzyme (2, 3). For the remaining nuclear-encoded (also referred to as cytoplasmic) polypeptides there is growing, though indirect, evidence suggesting a role as control units of the catalytic process. This hypothesis was first suggested by the tissue specificity of some cytoplasmic components of the enzyme (4) and later supported by the finding that different genes can code for alternative forms of the same subunit (5). Very recently a case where this subunit substitution occurs under physiological conditions has been documented (6). In spite of these results, the function of the cytoplasmic subunits of cytochrome c oxidase remains obscure.

Attempts to solve the problem through molecular genetics, either selectively depriving the enzyme of one or more of the smaller polypeptides or substituting them with copies containing “site-directed” mutations are in progress even though a difficult assembly of the complex could severely limit this approach (7-9). The depletion of few oxidase polypeptides has been obtained by chemical and/or physical treatments and used to suggest that the removed cytoplasmic components were unnecessary in the electron transfer and proton pumping activities of the purified complex (10-12). However, a major assumption in this kind of experiment is that a regulatory effect, if present, is exerted by and not through a subunit. In the latter case (modulation operated by a ligand upon binding to the subunit) activity measurements performed with the isolated enzyme (i.e. absence of the physiological cellular environment and hence of the hypothetical ligand) may be not adequate to show an effect.

Among the possible cellular modulators of cytochrome c oxidase, ATP is a good candidate since it is the main conversion product of the energy generated by the electron transfer chain and as such or complexed with Mg$^{2+}$ it is an effector of several enzymes. Moreover, it has already been reported that at physiological concentrations it markedly affects the kinetics of the interaction between cytochrome c and cytochrome c oxidase (12).

This work provides evidence that ATP, by interacting specifically with the cytoplasmic subunits IV and VIII, induces an allosteric conformational change that involves the acidic residues forming the cytochrome c binding site; as a consequence the enzyme-substrate interaction is modified depending on the nucleotide concentration. The specificity of this effect and the influence of the Mg$^{2+}$ ions are also investigated.

**EXPERIMENTAL PROCEDURES**

Enzyme Preparations—Beef heart cytochrome c oxidase was prepared according to Steffens and Buse (14) omitting the final dialysis step. Turkey (Meleagris gallopavo) and tuna (Thunnus thynnus thynnus) cytochrome c oxidases were purified from the heart tissue by the procedure of Bisson et al. (15).

Activity Measurements—Oxidase activity was measured polarographically in a water-jacketed chamber using a 4004YS1 oxygen electrode (Yellow Springs Instrument Co.). Assays were performed in 25 mM Tris-cacodylate, pH 7.6, in the presence of 3 mM ascorbate, 0.69 mM $N,N,N',N'$-tetramethyl-p-phenylenediamine, and 0.06% lauryl maltoside at 25°C. This detergent was substituted by egg yolk lecithin (0.02%) when oxidase lipidated by the method of Vik and Capaldi (16) was used. Routinely the concentration of cytochrome c oxidase was 22.8 nM while that of cytochrome c varied over a range of 0.02-30 μM. The 300-500 mM stock solutions of the different
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tnucleotides used for additions to the assay buffer were adjusted to pH 7.4 with Tris base.

Chemical Modification—Two water-soluble reagents, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) from Serva and 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-p-toluenesulfonate (CMC) from Fluka, were used for modification of the reactive carbonyl groups present on the enzyme surface. Radioactive labeling experiments were performed in the presence of the nucleophile [14C] glycine ethyl ester (52.2 mCi/mmol, New England Nuclear) essentially under the conditions previously described (17). Fluorographs were obtained by using Amplify™ (Amersham Corp.) and x-ray X-Omat films (Kodak). Radioactive gels were sliced (Kinto 1-mm thick slices, incubated with 200 μl of water-glycercol (2:1) for 2 h, and then over night with 400 μl of tissue solubilizer (Soluene 350, Packard Instrument Co.) at room temperature in tightly stoppered vials. After addition of 4 ml of the scintillation mixture and at least 24 h of further incubation the vials were counted. The 100 mM stock solution of nucleotides and phosphate used in these experiments was adjusted to pH 6.5 ± 2 °C with NaOH. Further details are reported in the figure legends.

Photoaffinity Labeling with Arylazido-Cytochrome c—Photoactivatable derivatives of horse heart cytochrome c were prepared by the procedure of Bisson et al. (18). The fraction containing cytochrome c modified at lysine 13 with the photo reactive nitroarylazido group was used for the experiments described in this work. Cytochrome c oxidase (4 μM final concentration) and the photo reactive substrate (12 μM) were ultracentrifuged in 25 mM Tris-cacodylate, pH 7.4, for 30 min at 70,000 rpm in 5 × 50-mm test tubes. The samples (20-100 μl in 5 × 50-mm test tubes) were illuminated for 20 min with a long-wave UV lamp (UL-56 Black Ray, Ultra-Violet Products, San Gabriel, CA) provided with a filter to cut off radiations below 300 nm. Oxidase was then directly analyzed for electron transfer activity.

Protein for SDS-PAGE was recovered by ultracentrifugation (6 h, 20,000 X g) of the samples brought to 100 mM NaF, pH 7.4, and loaded on a discontinuous gradient formed by 20 mM Tris-cacodylate, 5% sucrose, 200 mM NaCl, pH 7.4 (0.5 ml) and by 20 mM Tris-cacodylate, 10% sucrose, pH 7.4 (1 ml); this procedure allowed for the complete removal of the cytochrome c not covalently bound to the enzyme. All operations were performed at 2 °C.

Photoaffinity Labeling with [γ-32P]ATP—Oxidase (5 μM final concentration) and ATP (0.5 mM) were dissolved in 25 mM Tris-cacodylate, pH 7.4, and loaded on a discontinuous gradient formed by 20 mM Tris-cacodylate, 5% sucrose, 200 mM NaCl, pH 7.4 (0.5 ml) and by 20 mM Tris-cacodylate, 10% sucrose, pH 7.4 (1 ml); this procedure allowed for the complete removal of the cytochrome c not covalently bound to the enzyme. All operations were performed at 2 °C. The radioactive labeling of the different enzyme subunits was analyzed with autoradiography of the dried SDS-PAGE slab gel in cassettes with an intensifying screen.

Chemicals—Lauryl β-D-maltopyranoside and Tween 80 were obtained from Behring Diagnostics and Fluka, respectively. ATP, ADP, AMP disodium forms, and magnesium ATP were purchased from Sigma. ATP, ADP, AMP free acid, and UTP were products of Boehringer Mannheim. Other chemicals were from the sources indicated: acrylamide (Serva); methylenebis acrylamide (Kodak); cacodylic acid (Carlo Erba); tris(hydroxymethyl)aminomethane (Jansen).

Miscellaneous—Spectrometric determinations were performed on a Perkin-Elmer lambda 5 UV/vis spectrophotometer using the following extinction coefficients: cytochrome c, ε(550-630)nm = 29 mM cm −1; cytochrome c oxidase (dithionite reduced minus air oxidized), ε(500-650) = 24 mM cm −1. SDS-PAGE was performed according to either Kadenbach et al. (19) on 20 × 20-cm slab gels or Swank and Munkres (20) on 10 × 10-cm slab gels.

RESULTS

The kinetics of reaction of horse heart cytochrome c with detergent-solubilized bovine oxidase in the presence of differ-
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![Graph](image)

**Fig. 2.** Effect of the MgATP concentration on the kinetics of cytochrome c oxidase. [ ] 1 mM MgCl₂ and 9 (○), 6 (○), 3 (△), and 1 (○) mM MgATP, respectively; 1 mM MgCl₂ was present in the buffer. Assay conditions were as described in the legend to Fig. 1. Dashed line shows the control for comparison. TN, turnover number (mol of cytochrome c s⁻¹ (mol of AA₀)⁻¹).

The lowering of its concentration or the substitution with ADP, MgATP, AMP, or phosphate progressively reduces the degree of protection (Fig. 1).

The changes involved at the molecular level in the apparently correlated results of Figs. 1 and 3 may be clarified by studying the direct influence of ATP and of the other polyvalent phosphate anions on the carbodiimide-reactive carboxyl residues.

Fig. 4A shows the Coomassie Blue gel electrophoretic patterns of the enzyme after reaction with CMC in the presence of the different nucleotides and phosphate. Extensive modification under standard conditions (lane C) alters the electrophoretic migration of several enzyme subunits. This behavior may be attributed either to the derivatization of the exposed carboxyl groups and/or to possible inter- and intrasubunit cross-links generated with neighboring nucleophilic residues (23). As a consequence, in a high resolving SDS gel, such as that of Fig. 4, a modified polypeptide can appear either broader, or split in multiplets, or in a extreme case it can move to a different position, generally at higher molecular weight. All these events can be recognized in Fig. 4A by comparison of lane C with lane N (native enzyme). The presence of ATP (lane T) or ADP (lane D) in the reaction mixture generally reduces these modifications (but see below) while AMP (lane M) or phosphate (lane P) appears to be rather ineffective.

The distribution of the carboxyl groups capable of reacting, after carbodiimide activation, with the radioactive nucleophile [¹⁴C]glycine ethyl ester is shown in Fig. 4B. On the left are the labeling patterns obtained with CMC. Quite surprisingly subunit II, which is thought to contain the cytochrome c binding site (18, 24) and hence most of the acidic residues involved in the electrostatic interaction with the substrate (21), is heavily modified in all cases in spite of the large differences in the activities of the reacted samples. In the case of the two additional labeled polypeptides, subunits I and V (Buse's nomenclature), the bound radioactivity appears to correlate well with inhibition. ATP is the most potent in lowering the level of the counts associated to these subunits while AMP is totally ineffective (compare lanes D and M with lane T in Fig. 4B).

These results are confirmed by using the more reactive carbodiimide EDC (Fig. 4B, right). Again the radioactivity in subunit II does not show relevant differences in autoradiography, but three other cytoplasmic subunits in addition to those discussed above, namely VII, VIIIa, and VIIIb, reveal different labeling patterns. Only in one case a polypeptide, subunit VIIIb, appears more heavily labeled in the ATP-protected enzyme than in the control. It should be noticed, however, by comparison with the Coomassie Blue profiles reported in Fig. 4A that this latter subunit aggregates during modification in the absence of ATP or ADP; consequently evaluation of its labeling in the various conditions is virtually impossible.

The similar levels of counts found in subunit II from the different samples, despite large differences of enzymatic activity and the central role of this polypeptide in the binding of cytochrome c (17, 18, 21), were further investigated. Samples of the enzyme were treated with EDC-[¹⁴C]glycine ethyl ester in the presence or absence of 3 mM ATP under the standard conditions described in the legend to Fig. 4. After reaction, aliquots of the two samples were diluted to decrease the nucleotide concentration in the ATP "protected" enzyme...
to a negligible level, and again submitted to a new reaction cycle. The distribution of radioactivity among the different polypeptides was analyzed by slicing and counting the gels after SDS-PAGE. As shown in Fig. 5, the counts incorporated by subunit II are essentially the same in spite of the presence of 3 mM nucleotide and 500 \( \mu \)M \( [14C] \) glycine ethyl ester. B, right: corresponding labeling patterns obtained by substitution of CMC with 2.5 mM EDC. Abbreviations are the same as in A. Other reaction conditions are as reported in the legend to Fig. 3. Subunit nomenclature is from Buse et al. (39).

Further evidence in favor of an ATP-induced conformational change affecting the catalytic binding site is obtained by a completely different approach based on the use of a photoreactive analogue of horse heart cytochrome c (18). In this derivative the photoactivatable nitroarylazido group is linked to Lys-13 in the center of the binding site. On illumination in the presence of oxidase the azidoctytochrome c cross-links to oxidase subunit II generating a covalent inactive enzyme-substrate complex (18, 24).

ATP and the other nucleotides appear to affect strongly the yield of photoaffinity labeling. As shown in Fig. 6, the loss of activity found after the photochemical reaction is very similar to that of the control for AMP and phosphate, but it can be completely prevented by ATP. The electrophoretic profiles of the enzyme in the two extreme cases (Fig. 7) demonstrate that this protection is a consequence of a lack of cytochrome c cross-linking caused by ATP. The comparison of the data of Fig. 6 rules out a significant role of ionic strength and reveals striking similarities with the results obtained through chemical modification experiments (see Fig. 3).

A basic problem in this investigation concerns the location of the ATP binding site. A preliminary answer to the question has been reported very recently (25). A radioactive arylazido derivative of ATP was used in photolabeling experiments to show a specific cross-linking to two nucleo-coded polypeptides of bovine cytochrome c oxidase, namely IV and VIII. However, the nitrene, generated upon illumination of the

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**Fig. 4.** Effect of adenine nucleotides on the electrophoretic and labeling patterns of cytochrome c oxidase modified by water-soluble carbodimidines. A, Coomassie Blue-stained subunits of cytochrome c oxidase after treatment of the enzyme with 10 mM CMC and SDS-PAGE according to Ref. 19. Lane N is native oxidase; lane C shows the modification introduced by the reagent; the symbols T, D, M, and P identify the samples modified in the presence of 6 mM ATP, ADP, AMP, or 10 mM phosphate, respectively. B, left: autoradiographies of samples treated as in A in the presence of 3 mM nucleotide and 500 \( \mu \)M \( [14C] \) glycine ethyl ester. B, right: corresponding labeling patterns obtained by substitution of CMC with 2.5 mM EDC. Abbreviations are the same as in A. Other reaction conditions are as reported in the legend to Fig. 3. Subunit nomenclature is from Buse et al. (39).
azido moiety, does not exhibit the same reactivity versus the different amino acid residues. Depending on the chemical nature and orientation of the groups present in the binding site, the yield of cross-linking can show a large variation. For this reason highly reactive residues present on the protein surface could act as a sink toward the nitrene and generate cross-links in areas not related to the nucleotide binding site (26).

Radioactive ATP has been directly used as a photoaffinity label (27–30). This approach may not suffer from the above-mentioned limitation, but it has the disadvantage of requiring protein-damaging UV radiation to activate the nucleotide adenine ring. Nevertheless, if used in conjunction with complementary techniques and with appropriate controls, it can provide valuable information.

Fig. 8A shows the result obtained with bovine oxidase after illumination and SDS-PAGE. In agreement with the results obtained with the azido-ATP, subunits V and VIII are labeled (Fig. 8A, lanes 1, 2, and 5). No radioactivity is associated with the enzyme subunits when the sample is kept in the dark (Fig. 8A, lane 3) while when oxidase is irradiated under denaturing conditions (Fig. 8A, lane 4) most polypeptides are labeled. Magnesium ions reduce the extent of labeling (Fig. 8A, lane 1) while phosphate exerts only a minor effect even at a concentration 1 order of magnitude higher than that of ATP (Fig. 8A, lane 2).

The possibility that the existence of an ATP binding site is a general feature of eukaryotic cytochrome c oxidases was investigated by similar photoaffinity labeling experiments with the oxidase purified from bird (turkey, duck, chicken) and fish hearts (tuna, swordfish, torpedo). As an example the results obtained with oxidases from a bird (turkey) and a fish (tuna) are compared in Fig. 8B. Turkey and, more generally, bird oxidases show a distribution of radioactivity which is remarkably similar to that of the bovine enzyme. The labeling of tuna and the other fish oxidases is extremely weak, and only in overexposed autoradiographs some cross-linking is detectable. In no case was radioactivity significantly incorporated by the mitochondrial subunits I, II, and III.

**DISCUSSION**

Attempts to demonstrate a role for the cytoplasmic subunits of cytochrome c oxidase have been hampered so far by the lack of significant functional changes in structurally different forms of the same enzyme (5, 6, 10, 12). Perhaps a more direct approach to the problem is the investigation of the molecular features which are at the basis of a different behavior of oxidase under well defined circumstances. In this respect the effect of ATP on the "high affinity" $K_m$ of the enzyme is certainly attractive (13). Moreover, since the cytochrome c binding site of oxidase is necessarily involved, this study may be approached with the chemical modification and photoaffinity labeling techniques previously used to map this site (17, 18, 21, 22).

The linearization of the kinetics is not unique to ATP since UTP is even more efficient, suggesting that the density of the negatively charged phosphate groups present in the molecule is the governing factor more than the aromatic ring linked to the sugar moiety. This observation is supported by the fact that higher concentrations of ADP or magnesium-complexed...
ATP are required to produce a similar effect.

From the experimental data reported above an apparent $K_D$ of $2 \pm 5 \times 10^{-3}$ M for the binding of ATP to oxidase may be estimated. Attempts to determine directly the binding constant either by equilibrium dialysis or by ultracentrifugation were unsuccessful possibly because of changes in the aggregation state of the enzyme at the high concentrations needed for these experiments.

The relative low affinity and specificity of the nucleotide site(s) on oxidase does not mean, however, that the observed changes are simply the result of an increasing ionic strength (31). This is quite clear from the photolabeling experiments with bovine heart oxidase under the standard conditions reported under “Experimental Procedures” in the presence of: 1, 1 mM MgCl$_2$; 2, 5 mM sodium phosphate; 3, no light; 4, 1% SDS; 5, no additions. Lane 0 is the correspondent Coomassie Blue-stained pattern of the enzyme. Panel B reports the result obtained under standard conditions by labeling the enzyme purified from the hearts of: 3, turkey; 4, tuna. Lanes 1 and 2 are photographs of the correspondent stained gels. SDS-PAGE was performed as described by Kadenbach et al. (19).

The analysis of the labeling profiles (Figs. 4, 5 and 7) suggests that a conformational change of the enzyme is induced by ATP binding. This effect appears to propagate to the entire molecule though, as indicated by hydrophobic photolabeling experiments with $[^{32}P]3$-(trifluoromethyl)-3-(miodophenyl)diazirine (data not shown), it does not involve the protein surface exposed to lipids. Unexpectedly the rearrangement in the cytochrome $c$ binding site contributed by subunit II seems limited, and it may involve a simultaneous exposure and hiding of acidic residues. However, because of the relatively high experimental error, this latter conclusion remains to be tested at the sequence level. The quantitation of the data reported in Fig. 5 shows that a major effect is also exerted on the smaller subunits and that it can be reversed simply by lowering the ATP concentration.

Although there is a general consensus on the fact that subunit II contains the binding site for cytochrome $c$ (2, 3, 18, 21, 22), it has been shown that some of the cytoplasmic subunits can also contact cytochrome $c$ (17) and hence, at least in principle, contribute to its binding. The present experiments further support this hypothesis. On the other hand it should be considered that the ATP-induced disappearance of acidic residues from the protein surface and the concomitant reduction of the affinity for cytochrome $c$ are two events that, particularly in the case of a large molecule such as cytochrome $c$ oxidase, may not always be directly linked. This is demonstrated by subunit VII where one or more of these carboxyl groups resides; subunit VII can in fact be digested with trypsin with no alteration of the enzyme kinetics either in the presence or in the absence of ATP (data not shown).

Also the lack of cross-linking after photolabelling of azidocytocrome $c$ appears to be the consequence of the same conformational changes that ultimately cause the decrease in the affinity for the substrate observed kinetically.

The possibility that these results might simply reflect different binding properties of the polyanion anions either to the oxidase interacting site(s) with the substrate and/or to cytochrome $c$ deserves some comments. In the case of oxidase, a direct effect of negative ions, such as ATP, ADP, and phosphate, on the cluster of carboxyl residues forming the substrate binding site is clearly unlikely. On the other hand these anions bind to cytochrome $c$, but the strength of the interaction (13, 32) follows an order that is opposite to their ability of producing the effects described above (Figs. 1 and 6). Consequently the influence of ion binding to cytochrome $c$ should be almost negligible here (see also Ref. 13).

Photoaffinity labeling with radioactive ATP supports the idea of a specific binding site(s) in the oxidase which is conserved in the enzyme from different eukaryotic sources. In this context the absence of a significant cross-linking to the fish oxidases is explainable. As previously mentioned, unlike the triphosphate group, the photoactivatable adenine ring present in ATP does not appear to be essential to induce the effects described above; consequently, when bound to oxidase, it probably faces poorly conserved residues that in the case of the fish enzyme may also be poorly reactive or not properly oriented. Essentially for similar reasons the labeling of subunits IV and VII does not unequivocally localize the position of the triphosphate group that, at least in principle, could also be in contact with mitochondrial subunits. Therefore, although the present evidence based on the photoaffinity labeling of selected nuclear coded subunits and on the predominant location of the cytochrome $c$ binding site on subunit.
II favors the idea of an allosteric conformational change, further investigations are required to completely define these aspects of the interaction.

Moreover, the physiological relevance of the effect remains unclear. It has been suggested that ATP, by converting the oxidase to its low affinity form, causes a redistribution of cytochrome c from the oxidase to phospholipids since the $K_D$ of phospholipids is comparable to the $K_D$ for the low affinity site of the enzyme (13). In this respect the stoichiometry of the cytochrome c binding components present on the C-side of the mitochondrial inner membrane becomes relevant. Since every two molecules of cytochrome c there are two oxidases, this effect of oxidase to its low affinity form, causes a redistribution of the mitochondrial inner membrane becomes relevant. Since every two molecules of cytochrome c there are two oxidases, and about 10-30 cardiolipin molecules (33, 34), small changes in the enzyme affinity for the substrate may have important consequences on reaction rates. In general a lowering of ATP concentration should increase the enzyme-substrate interaction and hence modify the electron transfer rate.

Although one could argue that even other polyvalent anions may have important consequences on reaction rates. In general a lowering of ATP concentration should increase the enzyme-substrate interaction and hence modify the electron transfer rate.

Our experiments suggest that ATP is a possible allosteric effector of oxidase, but they do not exclude the possibility that other factors, present in the intracellular milieu, might simultaneously act on the in vivo enzyme leading to effects that are difficult to predict a priori and could even be different from the phenomena described above. Moreover, the physical context of the enzyme might also be very important in the light of recent experiments suggesting a possible involvement of the electrochemical potential gradient in the stabilization of different enzyme conformations (36). The hypothesis that ATP might participate in the regulation of respiration in higher organisms has been considered in a recent review (37).

Whether the ATP-cytochrome c oxidase interaction is physiologically relevant or not, the present data support the idea of a modulation of the enzyme activity operated via the cytoplasmic subunits. This acquires importance in the light of recent observations that suggest, in addition to the presence of tissue-specific isoenzymes (4), the existence of different developmentally regulated forms of cytochrome c oxidase (38). The extension of our studies to these different forms of the enzyme and, for comparison, to prokaryotic oxidases appears now a consequent further step in this research line. Perhaps this different way of looking at the enzyme may offer a portrait of cytochrome c oxidase closer to the in vivo system than the image we have acquired from conventional in vitro studies.

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