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Effects of the Lipophilic Biscation, *bis*-Pyridinium Oxime BP12, on Bioenergetics and Induction of Permeability Transition in Isolated Mitochondria

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ABSTRACT. In order to further investigate the mechanism of action of bridged lipophilic *bis*-pyridinium oximes previously observed to interfere with mitochondrial metabolism and to induce growth arrest and apoptosis in HeLa cells (Nocentini *et al.*, *Biochem Pharmacol* **53:** 1543–1552, 1997), we studied the effects of a *bis*-pyridinium oxime with a polymethylene chain N = 12 (BP12) on isolated rat liver mitochondria. Respiration in the absence of ADP with succinate plus rotenone as substrate was not affected after treatment with various concentrations of BP12 up to 10 μM, while the ADP-stimulated respiration was slowed down, with a parallel decrease in ATP synthesis. No effects of BP12 were detected on membrane potential, ATPase activity, and inorganic phosphate transport, but the adenine nucleotide translocase was inactivated and a permeability transition of the inner membrane was induced in the presence of calcium. These data suggest that mitochondrial impairment of ATP synthesis and the formation of the permeability transition pore may be responsible for apoptotic cell death already observed in cells treated with BP12. BIOCHEM PHARMACOL **59**;3:261–266, 2000.

KEY WORDS. *bis*-pyridinium oxime; mitochondria; oxidative phosphorylation

BPO,§ a family of molecules whose charged pyridinium moieties are linked by a linear polymethylene chain of variable length, were initially designed as reactivators of organophosphorous-inhibited acetylcholinesterase [1], and were later assayed for other biological effects in yeast and human cells [2–4]. It was shown that BPO are able to inhibit the growth of HeLa cells and cause cell death by an apoptotic process [4]. BPO toxicity is closely correlated to the length of the methylene chain joining the two pyridiniums, and the most hydrophobic of the studied compounds, BP12, is the most efficient. It was also shown that BPO, due to their positive charge and their lipophilicity, preferentially localize in mitochondria [2, 3] and that continuous exposure of HeLa cells to BP12 alters mitochondrial metabolism, as indicated by the decrease in oxygen consumption and ATP stores. It is worth noting that these compounds did not produce any degradative action on mitochondrial DNA in human cells [4]. It was concluded that the cytotoxic effect of BPO, interestingly, is modulable by the hydrophobicity of the molecule and likely results from alteration of the mitochondrial energetic metabolism, ultimately eliciting a programmed cell death process. These compounds can thus present a potential pharmacological interest, since the higher plasma and/or mitochondrial membrane potential of numerous tumoral cells versus normal cells should promote their selective uptake and retention in cancer cells and allow a chemotherapeutic treatment of certain types of cancer, as has been proposed for other lipophilic cations [5, and references therein]. The present study attempted to gain further insight into the mechanism of action of BPO, in particular in the light of recent developments concerning the role of mitochondria in cell death [6], by analyzing the damage induced by BP12 on the different steps in oxidative phosphorylation and the eventual induction of the PT of the inner mitochondrial membrane, as this last process is central to the apoptotic process [6].

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§ *Abbreviations:* BPO, *bis*-pyridinium oximes; BP12, *bis*-pyridinium oxime N = 12; $DIOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; PT, permeability transition; AdNT, adenine nucleotide translocase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, 4-morpholinepropanesulfonic acid; and Pi, inorganic phosphate.

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MATERIALS AND METHODS

Chemicals

All the reagents were of the purest available grade and used without further purification. BP12 was synthesized and purified according to a published procedure [1]. Mitochondria (0.5 mg protein/mL) suspended in the respiratory

medium (*vide infra*) were incubated for 2 min in the presence of BP12.

Preparation of Mitochondria and Measurement of Respiration

Rat liver mitochondria were isolated as previously described [7] and the final mitochondrial pellet was suspended in 0.25 M sucrose to give a concentration of 80–100 mg protein/mL, measured according to the method of Waddel and Hill [8]. The mitochondria (0.5 mg protein/mL) were placed in a chamber with magnetic stirring of the respiratory medium (200 mM sucrose, 30 mM Tris–MOPS, pH 7.4, 5 mM Pi, 1 mM EGTA–Tris). The chamber, a thermostatted (T = 25°) water-jacketed vessel, was fitted for oxygen measurement with a Clark electrode (Yellow Springs Instruments) connected to a recorder. Succinate (10 mM) plus 2 μg/mL rotenone was used as substrate and ADP-stimulated respiration was measured by addition of 0.3 mM ADP.

Measurement of Membrane Potential

The membrane potential was quantitatively determined with the fluorescent probe pyronin G according to Tomov [9]. This cationic dye is concentrated by the mitochondria according to the Nernst equation. When concentrated in the matrix, the formation of non-fluorescent aggregates occurs, and the fluorescence intensity of the mitochondrial suspension changes depending on the membrane potential. The fluorescence intensity of the suspension (excitation and emission wavelengths 510 and 565 nm respectively) is determined at the studied state (F_E) as well as at zero membrane potential (F_O) with a Perkin Elmer LS-5 fluorimeter. The membrane potential is calculated according to the formula

$$E_{mV} = -59 \lg \left[\delta \left(\frac{\varphi}{\sqrt{\frac{3.5 \times 10^{-4}\delta}{\pi}(1-\varphi)}} - 1 \right) \right]$$

where $\varphi = F_E/F_O$, δ is the ratio of the volume of the mitochondria to the total sample volume, and π is the pyronin G concentration in the sample. Measurements were performed using mitochondria (0.5 mg protein/mL) suspended in the respiratory medium containing 10 μM pyronin G and 5 μM rotenone, in the absence or presence of BP12. Succinate was added at the concentration of 10 mM to create the membrane potential.

Measurement of ATPase Activity

ATPase activity was followed by measuring the mitochondrial swelling which occurs in mersalyl-treated mitochondria added with ATP when ATP hydrolysis is elicited [10, 11]. Control or BP12-treated mitochondria (0.5 mg protein/mL) were suspended in the respiratory medium. After incubation for 1 min in the presence of mersalyl (20 nmol/mg protein), 2 mM ATP was added and after 1 min ATP hydrolysis was induced by addition of 1.30 μM FCCP. Mitochondrial swelling was followed spectroscopically by the absorbance changes at 540 nm.

Measurement of Phosphate Carrier Activity

Phosphate transport was measured by the classical swelling technique of Chappel and Haarhoff [12]. Aliquots (0.1 mg protein/mL) of mitochondrial suspension, untreated or treated with BP12, were added to 100 mM ammonium phosphate (NH_4Pi) pH 7.2 and absorbance at 540 nm was recorded continuously.

Measurement of AdNT Activity

Fluorimetric measurement of ADP/ATP efflux was performed with mitochondria (0.5 mg protein/mL) incubated in the absence or presence of BP12 in 2 mL of respiratory medium as described by Passarella *et al.* [13]. An ATP-detecting system, consisting of glucose (2.5 mM), hexokinase (1 E.U.), glucose-6-phosphate dehydrogenase (0.5 E.U.), and $NADP^+$ (0.2 mM), was added. The NADPH formation in the extra-mitochondrial phase, which reveals ATP appearance due to externally added ADP, was followed fluorimetrically. The exchange reaction was started by adding ADP. The pyridine nucleotide fluorescence change was continuously monitored with excitation and emission wavelengths of 334 and 456 nm, respectively. The initial value of intra-mitochondrial pyridine nucleotide was equalized to zero. Checks were made to verify that in the absence of $NADP^+$ no significant change in fluorescence occurred during the experiment. The rate of increase in fluorescence, expressed in arbitrary units as scale division/min, was obtained as a tangent to the initial part of the progress curve.

ATP Measurement

The rate of ATP synthesis was measured according to the method of Lowry *et al.* [14]. Aliquots were withdrawn from the mitochondrial suspension incubated in the respiratory medium with succinate as substrate within 1 min after addition of 1 mM ADP and were precipitated with 2.6% perchloric acid. After neutralization of the supernatant, ATP was measured by adding the ATP-detecting system described above.

Formation of Permeability Transition Pores

Mitochondria (0.5 mg protein/mL) were suspended in a medium containing 200 mM sucrose, 10 mM Tris–MOPS, pH 7.4, 5 mM succinate–Tris, 1 mM Pi, 10 μM EGTA–Tris, 2 μg/mL rotenone, and 3 μg/mL oligomycin. Mitochondrial swelling after Ca^{2+} addition was followed spectroscopically as the decrease in the absorbance of the mitochondrial suspension at 540 nm measured with an

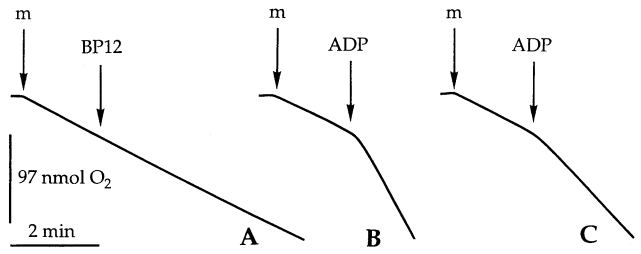

FIG. 1. Effect of BP12 on the rate of respiration. Mitochondria (0.5 mg protein/mL) were suspended in the respiratory medium (200 mM sucrose, 30 mM Tris–MOPS, pH 7.4, 5 mM Pi, 1 mM EGTA–Tris) in a thermostatted (T = 25°) vessel. Succinate (10 mM) plus 2 μg/mL rotenone was used as substrate. Oxygen consumption was followed with a Clark electrode connected to a recorder. State 4 respiration after addition of 10 μM BP12 (A). State 4 and state 3 respiration in control (B) and in 10 μM BP12 (C)-treated mitochondria. Where indicated (arrows), mitochondria (m), 10 μM BP12, and 0.3 mM ADP were added to the suspension.

SLM Aminco DW 2000 spectrophotometer operated in the split beam mode [15].

RESULTS

Effects on Respiration and ATP Synthesis

Respiration in the absence of ADP (state 4) was studied with succinate plus rotenone as substrate. Under these conditions, no effect of BP12 was detected when the isolated mitochondria were treated at various concentrations up to 10 μM (Fig. 1A). It is worthwhile noting that the oxygen consumption in mitochondria uncoupled with 1.3 μM FCCP (144 ± 3.8 natom O_2/mg/min) was not altered by BP12, suggesting that the transport of electrons to oxygen was not damaged (data not shown). Similar additions of BP12 did, however, slow down the ADP-stimulated respiration (state 3) (Fig. 1, B and C). The effects of increasing concentrations of BP12 on respiration in state 4 or 3 are represented in Fig. 2A, which shows that the rate of oxygen consumption in state 3 declined to 47% of the control value in the presence of 10 μM BP12. At the same time, ATP synthesis measured according to [14] was impaired and for 10 μM BP12 was 67% of the control value (Fig. 2B). The non-linear correlation between state 3 respiration and ATP synthesis is due to the fact that these functions were measured using different ADP concentrations (0.3 and 1 mM, respectively), while one step in oxidative phosphorylation involves a competitive type reaction with BP12 (see below). However, using 1 mM ADP, the residual oxygen consumption in state 3 in the presence of 10 μM BP12 was ~60%.

Action Mechanism Leading to ATP Synthesis Inhibition

As indicated above, state 4 respiration is not sensitive to BP12 addition. This fact suggests that the proton permeability of the inner mitochondrial membrane is not altered. We set out to verify this by using the fluorescent probe

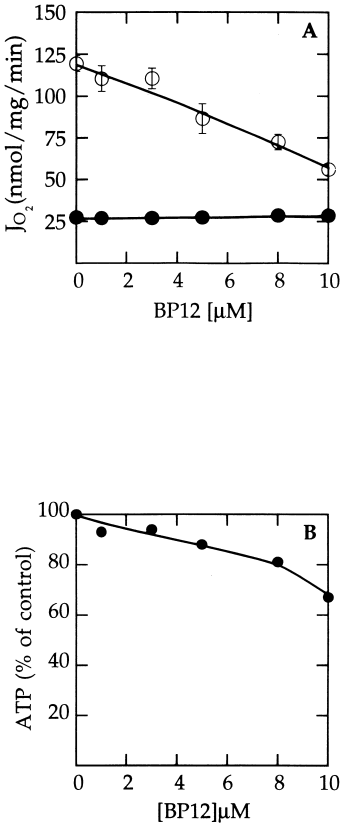

FIG. 2. Effect of BP12 on respiration (A) and ATP synthesis (B). Experimental conditions were as in Fig. 1. (A) Respiration (JO_2) of mitochondria in state 4 (●) or in state 3 (○) is plotted versus the BP12 concentration. (B) After a 2-min incubation in the presence of BP12, ATP synthesis versus BP12 concentration was measured 1 min after addition of 1 mM ADP according to the technique described in Materials and Methods. The final results are averages of at least three independent experiments.

pyronin G for the quantitative determination of the inner membrane potential according to the method of Tomov [9]. The mitochondria are de-energized with rotenone and the fluorescent intensity F_O of the suspension is recorded. After various additions of BP12, the mitochondria are energized by addition of succinate. The fluorescence declines to F_E and the membrane potential is calculated as indicated in Materials and Methods. When membrane potential is generated under such conditions, the pH difference on either side of the inner membrane is negligible and the membrane potential is in fact equal to $\Delta\mu_H+$, the electrochemical proton gradient. Indeed, the membrane potential ($\Delta\mu_H+$ = 190 mv) was not significantly modified by addition of BP12 even at the highest concentration of 10 μM. Therefore, the decline in oxidative phosphorylation can only result from effects on the ATPase complex and/or the phosphate compound carriers.

The effect of BP12 on ATPase activity was determined by the mitochondrial swelling method [10, 11]. Swelling induced by FCCP in ATP-added mitochondria, treated with mersalyl in order to inhibit the phosphate transport, was followed spectroscopically at 540 nm. No change in the

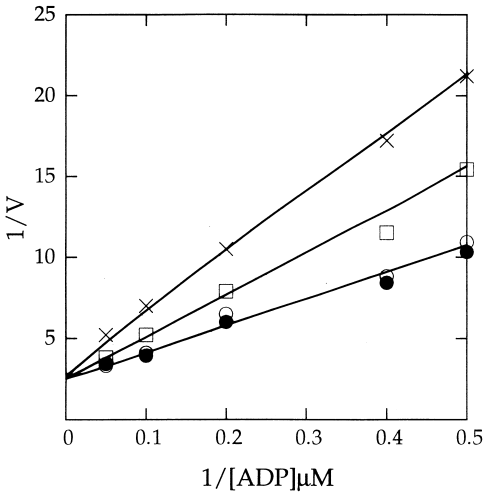

FIG. 3. Effects of BP12 on AdNT activity. Experimental conditions were as in Fig. 1. Carrier activity was followed fluorimetrically by detection of NADPH as reported in the text. The rate of exchange as a function of ADP concentration was determined in control (●) and in mitochondria treated with 1 (○), 5 (□), or 10 (X) μM BP12. The measurements were performed in duplicate in three different preparations.

rate of mitochondrial swelling was detectable after incubation of mitochondria with BP12 (data not shown), indicating that the ATPase complex is not inhibited by these compounds at the concentrations used in the present experiments.

Finally, the effects induced by BP12 on the oxidative phosphorylation process were examined at the level of the phosphate compound carriers, namely the inorganic Pi carrier and the AdNT. The transport of Pi measured by the swelling technique of Chappel and Haarhoff [12] was not impeded by addition of BP12, while the activity of the translocator AdNT, assessed by following spectroscopically the ATP efflux induced by externally added ADP in the presence of an ATP-detecting system, was impaired. Figure 3 shows the activity of the translocase as a function of ADP concentration for 1, 5, or 10 μM BP12 and indicates a competitive inhibition of the carrier. At 1 μM BP12, the activity of the carrier was equivalent to that of the control, while for 5 and 10 μM the residual activity was ~75% and ~50%, respectively. These results suggest that damage to the AdNT, rather than damage to the ATPase complex or to the Pi carrier, is responsible for the inhibition of oxidative phosphorylation observed in the presence of BP12.

Effects on the Formation of PT Pores

All the experiments reported above were performed in the absence of Ca^{2+} in the incubation medium. Under these conditions, BP12 was not able to induce the PT of the inner mitochondrial membrane because a certain level of Ca^{2+} loading is requisite to pore opening. However, a dramatic effect was observed in its presence. Indeed, the experiments reported in Fig. 4 show that control mitochondria loaded with 40 μM Ca^{2+} maintained their permeability barrier to sucrose, while addition of 3 μM BP12 induced permeabilization. The permeabilization was more rapid for higher concentrations of BP12, the process being prevented by the specific PT inhibitor cyclosporin A. The permeabilization increased with the calcium concentration (Fig. 5). In the absence of EGTA and oligomycin, the ATPase inhibitor, loading mitochondria with 10 or 5 μM Ca^{2+} induced the PT after additions of 3 or 10 μM BP12, respectively (Fig. 5, A and B). These experiments indicate that BP12 can induce pore opening at concentrations which do not perturb electron transport and ATPase complex activity, but do inhibit the activity of the AdNT translocator, thus affecting oxidative phosphorylation.

DISCUSSION

In a previous report, we indicated that mitochondria were likely the subcellular target for BPO cytotoxicity [4]. It was thus shown that BPO inhibition of cell growth, followed by apoptotic cell death, was associated with an impairment of oxygen consumption and a depletion of ATP intracellular stores. As no modification of mitochondrial DNA content relative to nuclear DNA content could be detected, it was suggested that a perturbation of mitochondrial bioenergetics was involved in the cytotoxic action of these compounds.

In the present report, using isolated rat liver mitochon-

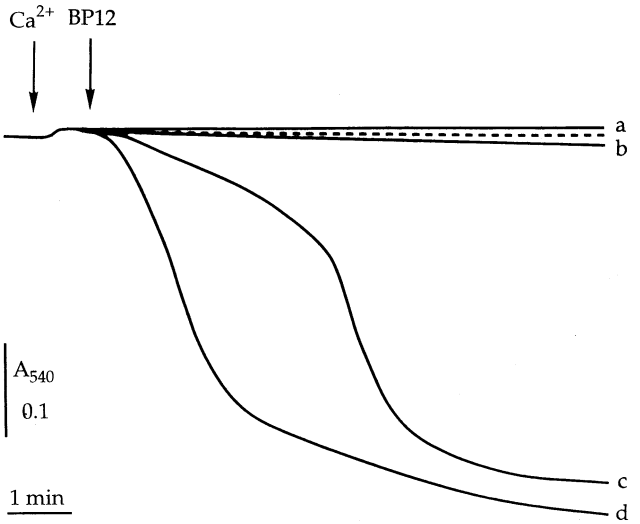

FIG. 4. Effects of BP12 on PT formation. The incubation medium contained 200 mM sucrose, 10 mM Tris–MOPS, pH 7.4, 5 mM succinate–Tris, 1 mM Pi, 10 μM EGTA–Tris, 2 μg/mL rotenone, and 3 μg/mL oligomycin. PT formation was followed spectroscopically at 540 nm. The experiments were started by addition of 0.5 mg/mL mitochondria (not shown). Trace a: control mitochondria; traces b, c, and d: BP12-treated mitochondria. Where indicated (arrows), 40 μM Ca^{2+} and 1, 3, or 10 μM BP12 (traces b, c, and d, respectively) were added to the suspension. Dashed line represents mitochondria treated with 10 μM BP12 in the presence of 0.8 μM cyclosporin A.

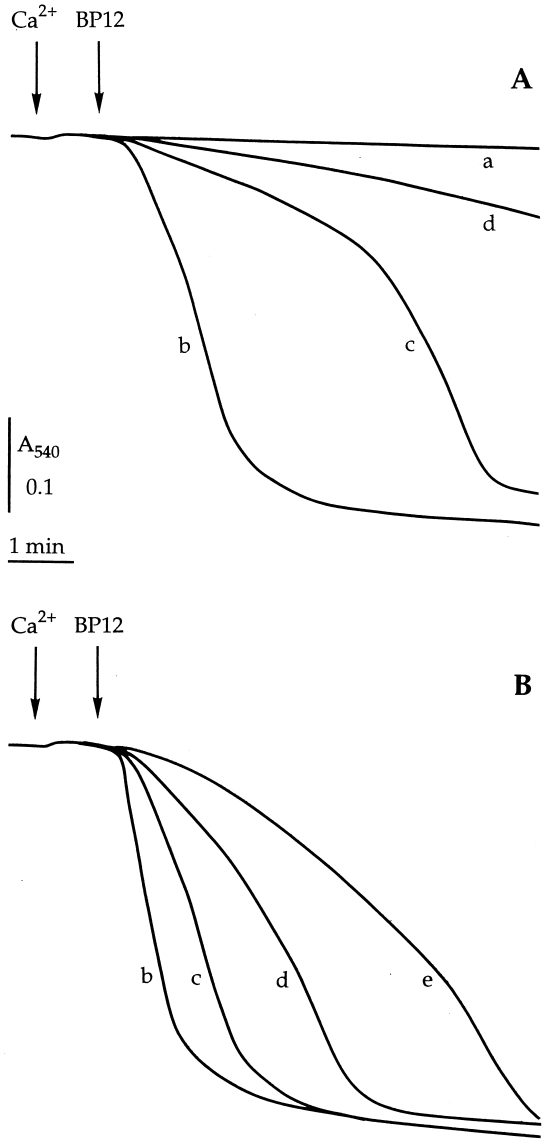

FIG. 5. Effects of BP12 on PT formation as a function of Ca^{2+} loading. Experimental incubation conditions were as in Fig. 4 except that EGTA and oligomycin were omitted. Mitochondria were incubated in the presence of 3 μM (A) or 10 μM (B) BP12. Where indicated (arrows), 40, 20, 10, or 5 μM Ca^{2+} (traces b, c, d, and e, respectively) and BP12 were added. Trace a represents control mitochondria loaded with 40 μM Ca^{2+}.

dria, we have investigated the effects of the compound in the series with the higher number (N = 12) of methylene groups in the linking chain, BP12, which is the most active on HeLa cells [4]. Interestingly, the oxygen uptake of isolated mitochondria in state 4 or uncoupled with FCCP was not affected by BP12, indicating that electron transport along the succinate pathway is not perturbed. However, state 3 respiration and oxidative phosphorylation were severely impaired at a concentration of 10 μM BP12. A more detailed study on the different steps of the oxidative phosphorylation process indicates that neither the membrane potential nor the ATPase and Pi carrier activity was affected. At variance, the AdNT activity was inhibited by BP12. This fact explains the effects on oxidative phosphorylation, because AdNT has great control over the rate of phosphorylation [16].

In intact BPO-treated cells, where mitochondria are not in pure state 4 or 3, the decrease in oxygen consumption and ATP synthesis can be easily explained by the decrease in state 3 respiration and AdNT activity observed with isolated mitochondria. We previously observed [4] that uptake of the cationic fluorochrome $DiOC_6(3)$ is increased in BPO-treated HeLa cells, and we hypothesized that BPO could increase the mitochondrial membrane potential by inhibiting the activity of the ATPase complex as reported for oligomycin [17]. In the present study using isolated organelles, we demonstrate that BP12 acts at a different level. However, a non-coherent behaviour of $DiOC_6(3)$, when used at concentrations of 40–100 nM, has been observed for the measurement of mitochondrial membrane potential with flow cytometry [6, 18, 19]. Consequently, in order to explain the previous observation in intact cells, it may be supposed that the increased fluorescent signal of $DiOC_6(3)$ at a concentration of 100 nM may be due either to its binding to other cellular membranes or to changes in plasma membrane potential occurring in BPO-treated cells, leading to a lower concentration of intracellular dye reducing the possible quenching.

The mitochondrial damage induced by BP12 is modulated by the calcium level in the medium. In its presence, low concentrations of BP12 can induce the PT of the mitochondrial membrane and therefore inhibit oxidative phosphorylation. In its absence, it is well known that PT cannot occur, whatever the inducer [20]. However, BP12 also interacts with AdNT, a complex with a large control coefficient over oxidative phosphorylation [16]. Both mechanisms could be united to reduce the ATP stores in intact cells while pore opening leads the cells to apoptosis.

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