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Calcium-Dependent Mitochondrial Formation of Species Promoting Strand Scission of Genomic DNA in U937 Cells Exposed to *tert*-Butylhydroperoxide: The Role of Arachidonic Acid

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Treatment of U937 cells with a sublethal concentration of *tert*-butylhydroperoxide generates DNA single strand breakage in U937 cells and this response is increased by caffeine, ATP, pyruvate or antimycin A. As we previously reported (Guidarelli, Clementi, Brambilla and Cantoni, (1997) Biochem. J. 328, 801–806), the enhancing effects of antimycin A are mediated by inhibition of complex III and the ensuing formation of superoxides and hydrogen peroxide in a reaction in which ubisemiquinone serves as an electron donor. Active electron transport was required in pyruvate-supplemented cells since the increased genotoxic response occurred as a consequence of enforced mitochondrial Ca^{2+} accumulation, a process driven by the increased electrochemical gradient. The enhancing effects of caffeine or ATP were also the consequence of mitochondrial Ca^{2+} accumulation but these responses were independent on electron transport. The increased formation of DNA lesions resulting from exposure to *tert*-butylhydroperoxide associated with the Ca^{2+}-mobilizing agents or the respiratory substrate was mediated by arachidonic acid generated by Ca^{2+}-dependent activation of phospholipase A_2. Melittin, a potent phospholipase A_2 activator, and reagent arachidonic acid mimicked the effects of caffeine, ATP or pyruvate on the *tert*-butylhydroperoxide-induced DNA single strand breakage.

Keywords: *tert*-Butylhydroperoxide; DNA damage; arachidonic acid; calcium ion; phospholipase A_2; mitochondria

INTRODUCTION

tert-Butylhydroperoxide (tB-OOH) is an organic hydroperoxide that has been frequently utilized to mimick the effects of physiological short-chain lipid hydroperoxides[1–12]. In cultured mammalian cells tB-OOH effectively induces DNA single strand breakage[1–7] and this response is insensitive to antioxidants[1,5–7] but prevented by iron chelators.[1,5–7] In a recent study we demonstrated that nontoxic concentrations of tB-OOH enhance the intracellular concentration of free calcium ions ($[Ca^{2+}]_i$) and that, under these conditions, mitochondrial calcium uptake is a major route for clearance of the released calcium ions.[7] Further characterization of this response indicated that tB-OOH mobilizes Ca^{2+} from pools that are different from the endoplasmic reticulum-located, sarcoplas-

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mic/endoplasmic reticulum Ca^{2+} – ATPase-containing, inositol 1, 4, 5 – trisphosphate (IP_3)- and ryanodine (Ry)-sensitive Ca^{2+} stores. We found that at least part of the tB-OOH-derived DNA-damaging species are generated at the level of mitochondria via a Ca^{2+}-dependent process. Agents mobilizing Ca^{2+} from Ry-[7] or IP_3-sensitive[8] Ca^{2+} stores further increased the tB-OOH-induced DNA-damaging response as a consequence of the enforced mitochondrial accumulation of the cation. NADH-linked substrates also promoted an increased accumulation of Ca^{2+} in the mitochondria and, as observed with the Ca^{2+}- mobilizing agents, this event was associated with an enhanced formation of tB-OOH-derived DNA-damaging species.[9,10]

The genotoxic response evoked by this organic hydroperoxide is also enhanced by *bona fide* complex III inhibitors (e.g. antimycin A or 2-heptyl-4-hydroxyquinoline *N*-oxide[11]) or by low levels of exogenous nitric oxide leading to inhibition of complex III.[12] Under these conditions, electrons are directly transferred from ubisemiquinone to oxygen with concomitant formation of superoxides and hydrogen peroxide. These reactions are Ca^{2+}-dependent and the complex III inhibitors, or nitric oxide, did not affect the rise in $[Ca^{2+}]_i$ or the mitochondrial accumulation of the cation mediated by tB-OOH.

The above findings collectively suggest the existence of two separate mechanisms which may modulate the formation of lesions of genomic DNA in cells exposed to tB-OOH. One mechanism encompasses the mitochondrial formation of hydrogen peroxide which then migrates to the nucleus and generates the DNA-damaging hydroxyl radicals via interaction with chromatin-bound divalent iron. The other mechanism is of an as yet unknown nature, takes place at the mitochondrial level and is dependent on enforced accumulation of Ca^{2+}.

In the present study we investigated the nature of the latter mechanism. The results obtained indicate that arachidonic acid (AA) plays a pivotal role in the formation of free-radical intermediates that generate DNA lesions in cells challenged with tB-OOH and agents promoting enforced mitochondrial Ca^{2+} accumulation.

MATERIALS AND METHODS

Materials

Pyruvate, caffeine (Cf), ATP, rotenone, myxothiazol, antimycin A, ruthenium red (RR), Ry, melittin, AA, mepacrine, 4-bromophenacil bromide (BPB), 5,8,11,14-eicosatetraynoic acid (ETYA), tB-OOH and the remaining chemicals were from Sigma-Aldrich, Milano, Italy. RPMI 1640 culture medium was from GIBCO, Grand Island, NY, USA and fetal bovine serum, penicillin and streptomycin were from Seralab, Sussex, UK. T-75 tissue culture flasks were purchased from Corning, Corning, NY, USA. [methyl-^{14}C]-thymidine and polycarbonate filters were obtained from NEN/Dupont, Boston, MA, USA and Nuclepore, Pleasanton, CA, USA, respectively.

Cell culture and treatments

Human myeloid leukemia U937 cells were cultured in suspension in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 µg/ml), at 37°C in T-75 tissue culture flasks in a humidified atmosphere of 95% air-5% CO_2.

Respiration-deficient U937 cells were isolated by culturing the cells for 6 days in RPMI medium containing 400 ng/ml ethidium bromide, 110 µg/ml pyruvate and 5 µg/ml uridine, with medium changes at days 2 and 4.

Stock solutions of tB-OOH, pyruvate, ATP, RR, mepacrine and AA were freshly prepared in saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose). Myxothiazol, rotenone, antimycin A and Ry were dissolved in 95% ethanol. BPB and ETYA were dissolved in

dimethyl sulfoxide. Cf and melittin were dissolved in water or 5% acetic acid, respectively. At the treatment stage the final ethanol or dimethyl sulfoxide concentrations were never higher than 0.05%. Under these conditions ethanol or dimethyl sulfoxide was neither toxic nor DNA-damaging, nor did it affect the cyto-genotoxic properties of tB-OOH.

Measurement of DNA single strand breakage by alkaline elution

Cells were labelled overnight with [methyl-^{14}C]-thymidine (0.05 μCi/ml) and incubated for a further 6 h in a medium containing unlabelled thymidine (1 μg/ml). After treatments, the cells were analyzed for DNA damage by the alkaline elution assay, using a procedure virtually identical to that described in[13] with minor modifications.[14]Briefly, 3.5–4 × 10^5 cells were gently oaded onto 25 mm, 2 μm pore polycarbonate filters and then rinsed twice with 10 ml of ice-cold saline A containing 5 mM ethylenediaminetetraacetic acid (EDTA, disodium salt). Cells were lysed with 5 ml of 2% sodium dodecylsulfate, 0.025 M EDTA (tetrasodium salt), pH 10.1. Lysates were rinsed with 7 ml of 0.02 M EDTA (tetrasodium salt) and the DNA was eluted overnight in the dark with 1.5% tetraethyl ammonium hydroxide/0.02 M EDTA (free acid)/0.1% sodium dodecylsulfate (pH 12.1), at a flow rate of ca. 30 μl/min. Fractions were collected at 2 h intervals and counted in 7 ml of liquid scintillation fluid containing 0.7% glacial acetic acid. DNA remaining on the filters was recovered by heating for 1 h at 60°C in 0.4 ml of 1 N HCl followed by the addition of 0.4 N NaOH (2.5 ml) and was again determined by scintillation counting. DNA was also recovered from the interior of the membrane holders after vigorous flushing with 3 ml of 0.4 N NaOH. This solution was processed for scintillation counting as described above. Strand scission factor values were calculated from the resulting elution profiles by determining the absolute log. of the ratio of the percentage of DNA retained in the filters of the drug-treated sample to that retained from the untreated control sample (both after 8 h of elution).

RESULTS

DNA cleavage induced by tB-OOH is enhanced via electron transport-dependent and -independent mechanisms

A 30 min exposure of U937 cells to 200 μM tB-OOH does not produce obvious signes of toxicity. Cell death, as measured by trypan blue or lactate dehydrogenase assays, was never detectable immediately after the peroxide exposure or after up to 48 h of post-treatment incubation. This treatment, however, led to formation of DNA single strand breaks (Figure 1). The DNA-damaging response response mediated by the hydroperoxide was significantly augmented by the calcium-mobilizing agents Cf (10 mM) and ATP (1 mM), by the respiratory substrate pyruvate (5 mM) and by the complex III inhibitor antimycin A (1 μM; Figure 1). Rotenone (0.5 μM) as well as myxothiazol (5 μM) abolished the enhancing effects mediated by pyruvate or antimycin A and similar results were obtained using different respiratory substrates (e.g. β-hydroxybutyrate) or complex III inhibitors (e.g. 2-heptyl-4-hydroxyquinoline N-oxide; not shown).

Since rotenone and myxothiazol inhibit complex I and III, respectively, these results indicate that the mechanism whereby respiratory substrates or antimycin A enhance DNA strand scission caused by tB-OOH requires electron transport through complex I. This conclusion is emphasized by the results reported in Figure 1 which indicate that neither pyruvate nor antimycin A was able to increase DNA strand scission induced by tB-OOH in respiration-deficient U937 cells.

Figure 1 also shows that rotenone or myxothiazol had hardly any effect on the DNA cleavage

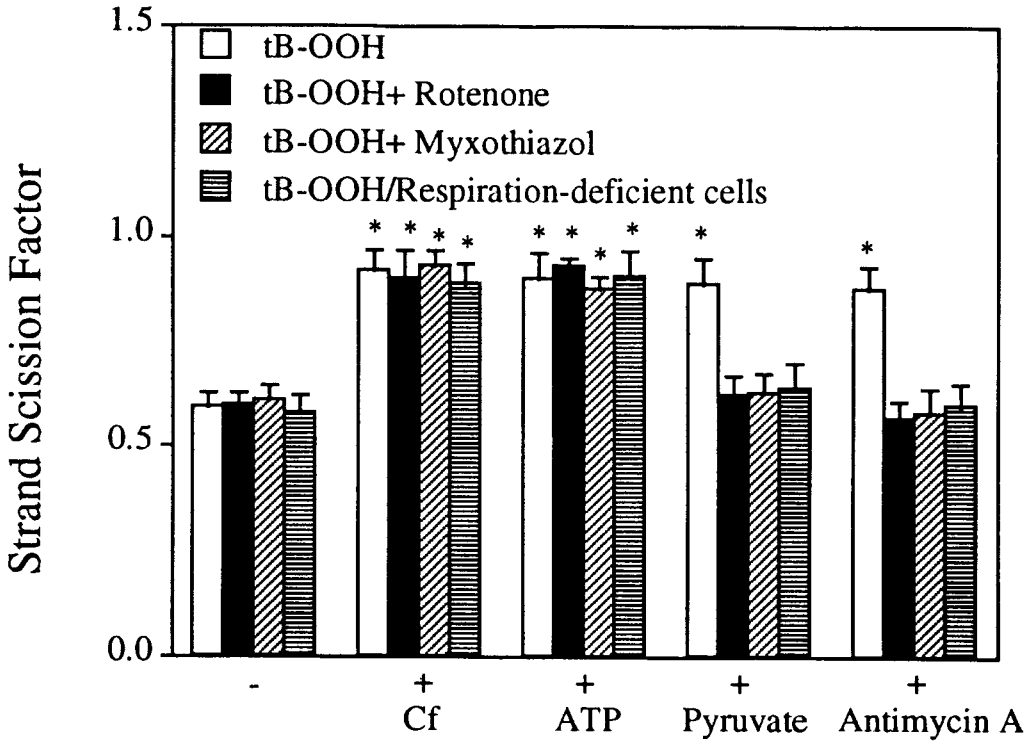

FIGURE 1 DNA cleavage induced by tB-OOH is enhanced via electron transport-dependent and -independent mechanisms. Respiration-proficient or – deficient cells were exposed for 5 min in saline A to 0 or 10 mM Cf, 1 mM ATP, 5 mM pyruvate or 1 μM antimycin A, and then treated for a further 30 min with 200 μM tB-OOH. 0.5 μM rotenone or 5 μM myxothiazol was added 5 min before Cf, ATP, pyruvate, antimycin A or tB-OOH. Treatment with antimycin A, Cf, ATP, or pyruvate in the absence or presence of myxothiazol or rotenone did not produce DNA single strand breakage. The level of DNA single strand breaks was measured immediately after the treatments using the alkaline elution technique. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments, each performed in duplicate, and were significantly different from those for DNA damage generated by the peroxide alone at $^{*}p < 0.001$ (unpaired *t* test)

caused by tB-OOH alone or associated with Cf or ATP. Furthermore, both calcium-mobilizing agents potentiated the genotoxic response evoked by tB-OOH in respiration-deficient cells as effectively as in respiration-proficient cells and the two cell types were equally sensitive to tB-OOH alone.

The effect of inhibitors of arachidonate metabolism

We studied the effects of the phospholipase A_2 (PLA_2) inhibitors mepacrine, BPB and ETYA on the DNA strand scission induced by tBOOH alone or combined with the calcium-mobilizing agents, respiratory substrates or complex III inhibitors. As illustrated in Figure 2, each of the PLA_2 inhibitors reduced the enhancing effects promoted by Cf, ATP or pyruvate in a dose-dependent manner. These effects were suppressed by as low as 5 μM mepacrine (A), 10 μM BPB (B) or 30 μM ETYA (C). In addition, mepacrine abolished the enhancing effects promoted by Cf or ATP in respiration-deficient cells (Table I). As reported in Figure 3, RR (25 μM) or Ry (20 μM) also reduced the DNA cleavage generated by tB-OOH associated with Cf, ATP or pyruvate and these responses were not further mitigated by mepacrine.

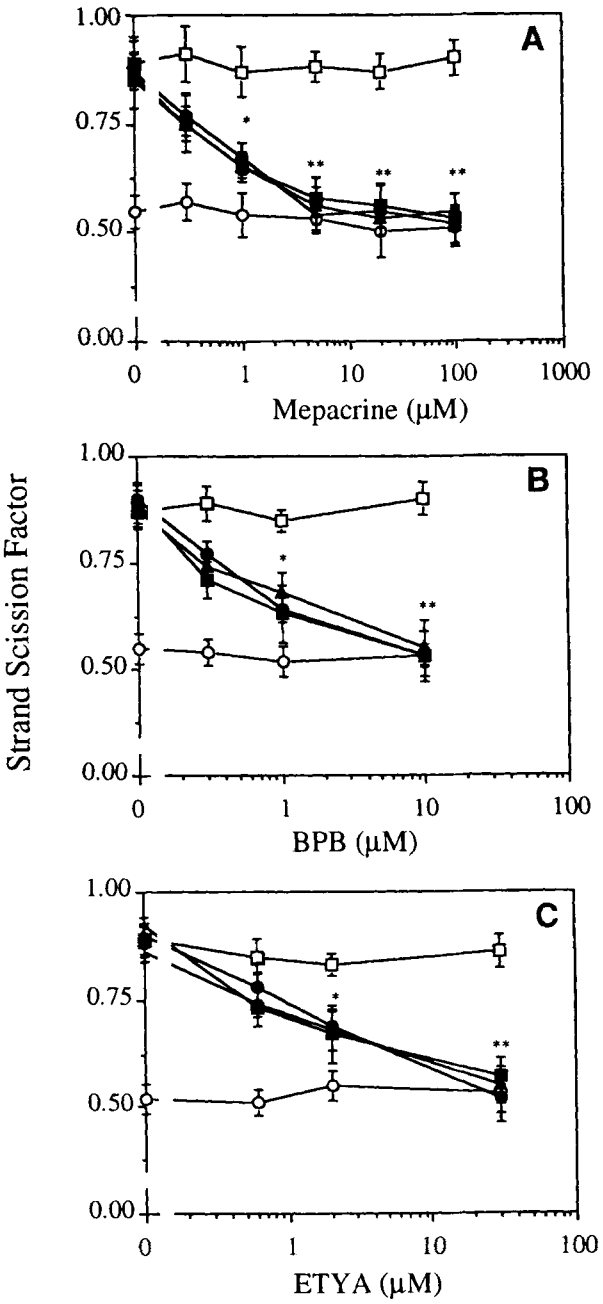

FIGURE 2 Effect of phospholipase A_2 inhibitors on the DNA strand scission induced by tB-OOH in antimycin A-, caffeine-, ATP- or pyruvate-supplemented U937 cells. Cells were exposed for 5 min in saline A to increasing concentrations of mepacrine (A), BPB (B) or ETYA (C), for an additional 5 min to 1 μM antimycin A (open squares), 10 mM Cf (closed circles), 1 mM ATP (closed squares), 5 mM pyruvate (closed triangles) or vehicol (open circles) and then treated for a further 30 min with 200 μM tB-OOH. The level of DNA single strand breaks was measured immediately after the treatments using the alkaline elution technique. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments, each performed in duplicate, and were significantly different from those for DNA damage generated by the peroxide associated with Cf, ATP, pyruvate or antimycin A at $^{*}p< 0.05$; $^{**}p< 0.01$ (Dunnett's test)

TABLE I Caffeine, or ATP, enhances DNA single strand breakage induced by tB-OOH in respiration-deficient cells and the inhibitor of phospholipase A_2 mepacrine prevents these responses

Treatment	*Strand Scission Factor*
200 μM tB-OOH	0.59 ± 0.06
200 μM tB-OOH + 10 mM Cf	0.92 ± 0.07*
200 μM tB-OOH + 1 mM ATP	0.90 ± 0,05*
200 μM tB-OOH + 5 μM Mepacrine	0.58 ± 0.04
200 μM tB-OOH + 5 μM Mepacrine + 10 mM Cf	0.61 ± 0.09
200 μM tB-OOH + 5 μM Mepacrine + 1 mM ATP	0.56 ± 0.05

Respiratory-deficient cells were exposed for 5 min to 0 or 10 mM Cf or 1 mM ATP and then treated for a further 30 min with tB-OOH. Where indicated, mepacrine was added to the cultures 5 min prior to Cf, ATP or tB-OOH. Treatment with Cf, ATP or mepacrine did not produce DNA cleavage. The level of DNA strand scission was measured immediately after the treatments using the alkaline elution technique. Results represent the mean ± S.E.M. calculated from 3–4 separate experiments and were significantly different from those for DNA damage generated by tB-OOH alone at *$p < 0.001$ (unpaired *t* test).

In contrast with the above results, none of the PLA_2 inhibitors utilized in these studies was able to reduce the DNA strand scission caused by tB-OOH alone or associated with antimycin A (Figure 2 A-C). The data illustrated in Figure 3 are consistent with our previous findings[11] and indicate that RR, unlike Ry, reduced the DNA cleavage generated by tB-OOH alone or associated with antimycin A.

Arachidonate as well as melittin enhance DNA strand scission caused by tB-OOH

The above experiments demonstrate that inhibitors of PLA_2 prevent the enhancing effects of Cf, ATP and pyruvate on DNA strand scission caused by tB-OOH. This would suggest that the PLA_2 product AA is a critical intermediate leading to formation of the tB-OOH-derived DNA-damaging species. If so, then AA should potentiate the DNA cleavage generated by tB-OOH. Indeed exogenously added AA, while not in and of itself DNA-damaging, potentiated the genotoxic response evoked by the hydroperoxide in a dose-dependent manner (Figure 4A). The enhancing effects were apparent using concentrations as low as 0.5 μM; 5 μM AA was as effective as 10 mM Cf, 1 mM ATP or 5 mM pyruvate in increasing the level of DNA single strand breakage caused by tB-OOH. At higher concentrations, AA did not further increase DNA strand scission. The effects of AA were insensitive to mepacrine or Ry but sensitive to RR (Figure 4B).

Melittin (0.03 μM), which stimulates PLA_2, also failed to generate DNA cleavage (not shown) but potentiated the genotoxicity of tB-OOH (Figure 4B) and this response was both prevented by mepacrine and insensitive to Ry. As observed with AA, the enhancing effects mediated by melittin were identical to those observed with the calcium-mobilizing agents or the respiratory substrate.

Finally, neither AA nor melittin further increased DNA damage in Cf-, ATP- or pyruvate-supplemented cells (not shown).

DISCUSSION

Although increases in mitochondrial Ca^{2+} have been demonstrated to enhance the extent of cleavage of genomic DNA caused by tB-OOH in U937 cells,[7–10] the mechanisms that trigger the mitochondrial formation of these DNA-damaging species have not been clearly defined.

In the present study we report clear experimental evidence indicating that the site of forma-

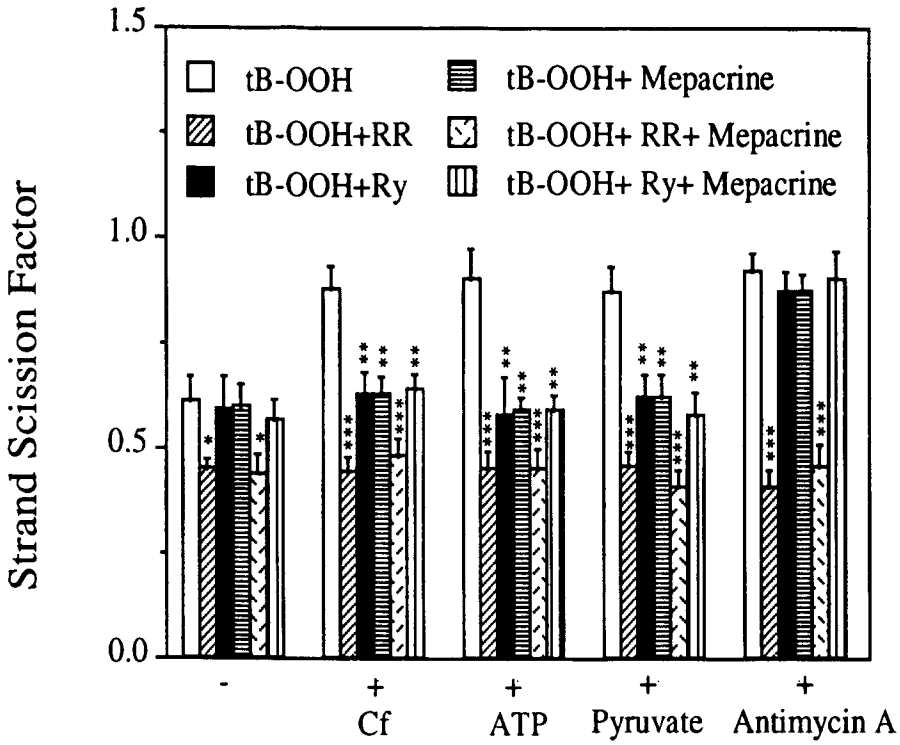

FIGURE 3 Effect of mepacrine on the DNA strand scission induced by tB-OOH, in the absence or presence of ruthenium red or ryanodine, in antimycin A-, caffeine-, ATP- or pyruvate-supplemented U937 cells. Cells were exposed for 5 min in saline A to 0 or 10 mM Cf, 1 mM ATP, 5 mM pyruvate or 1 μM antimycin A and then treated for a further 30 min with 200 μM tB-OOH. 5 μM mepacrine, 25 μM RR or 20 μM Ry were added 5 min before Cf, ATP, pyruvate, antimycin A or tB-OOH. Treatment with antimycin A, Cf, ATP, or pyruvate in the absence or presence of mepacrine, RR or Ry did not produce DNA single strand breakage. The level of DNA single strand breaks was measured immediately after the treatments using the alkaline elution technique. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments, each performed in duplicate, and were significantly different from those for DNA damage generated by the peroxide alone or associated with Cf, ATP, pyruvate or antimycin A at $^{*}p< 0.01$; $^{**}p< 0.001$; $^{***}p< 0.0001$ (unpaired *t* test)

tion of DNA-damaging species in cells exposed to tB-OOH and Cf or ATP is distal from the respiratory chain. This conclusion is supported by the observations that the enhancing effects mediated by the Ca^{2+} mobilzing agents were both insensitive to inhibitors of electron transport and apparent also in respiration-deficient cells (Figure 1).

These results are in contrast with those observed with pyruvate or antimycin A. The requirement for a functional respiratory chain is obvious in the case of antimycin A. Indeed, our previous work[11] demonstrated that the antimycin A-mediated enhancement of the tB-OOH-induced DNA-damaging response is the result of a sequence of events in which a) electrons are directly transferred from ubisemiquinone to molecular oxygen, b) superoxides formed in these reactions dismutate to hydrogen peroxide, c) the oxidant migrates to the nucleus and generates hydroxyl radicals, the final DNA-damaging species, by interacting with chromatin-bound transition metals. Thus, the respiratory chain is the site in which the DNA-damaging species are being formed and, not surprisingly, we found that the enhancing

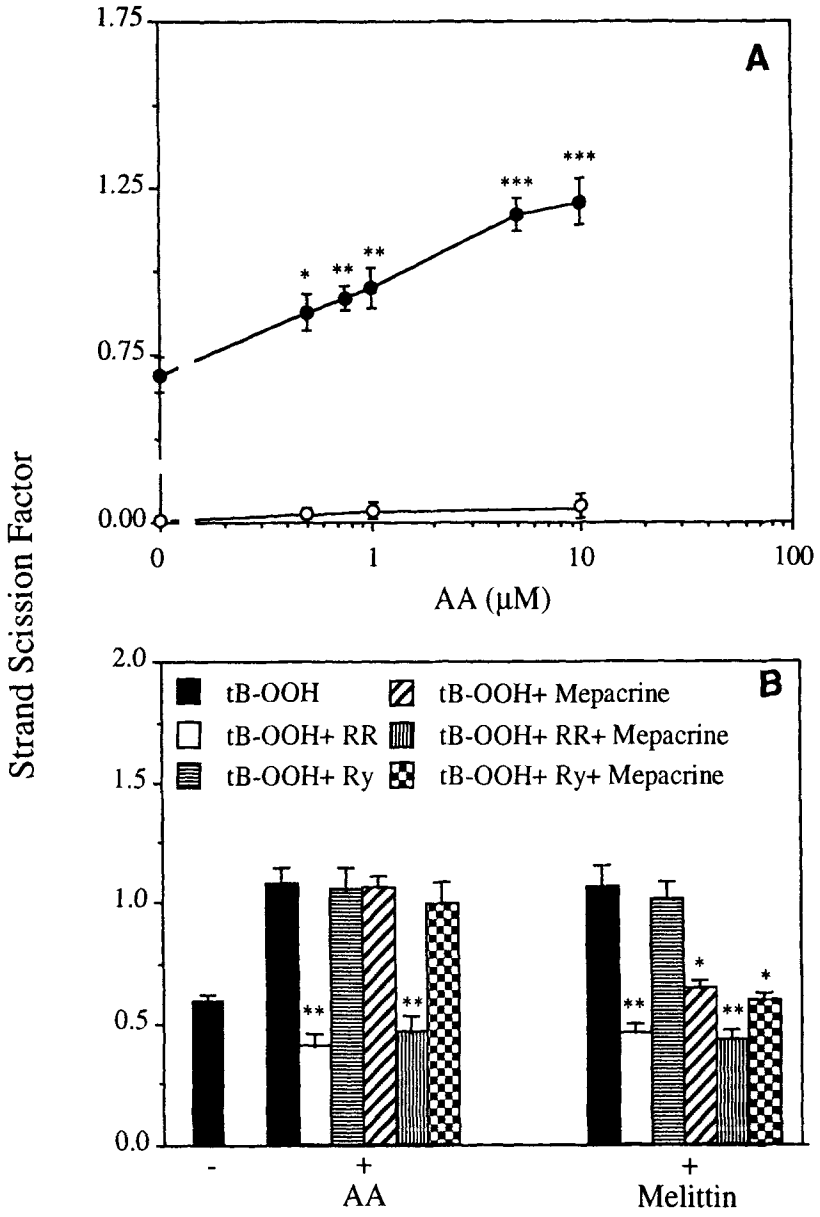

FIGURE 4 Arachidonic acid, or melittin, enhances DNA single strand breakage induced by tB-OOH. A, Cells were exposed for 5 min in saline A to increasing concentrations of AA and for additional 30 min to 200 μM tB-OOH (closed circles). The effect of increasing concentrations of AA alone was also investigated (open circles). The level of DNA single strand breaks was measured immediately after the treatments using the alkaline elution technique. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments, each performed in duplicate, and were significantly different from those for DNA damage generated by tB-OOH alone at $^{*}p< 0.03$; $^{**}p< 0.01$; $^{***}p< 0.001$ (unpaired *t* test). B, Cells were exposed for 5 min to 0 or 5 μM mepacrine, 25 μM RR or 20 μM Ry for an additional 5 min to 10 μM AA or 0.03 μM melittin and then treated for a further 30 min with 200 μM tB-OOH. Treatment with AA or melittin in the absence or presence mepacrine, RR or Ry did not produce DNA single strand breakage. The level of DNA single strand breaks was measured immediately after the treatments using the alkaline elution technique. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments, each performed in duplicate, and were significantly different from those for DNA damage generated by tB-OOH associated with AA or melittin at $^{*}p< 0.01$; $^{**}p< 0.001$ (unpaired *t* test)

effects of antimycin A were abrogated by either rotenone (which inhibits complex I), or by myxothiazol (which prevents the formation of ubisemiquinone) and were not apparent in respiration-deficient cells (Figure 1).

The fact that rotenone, myxothiazol and the respiration-deficient phenotype promoted similar effects after exposure to pyruvate may imply that also under these experimental conditions the DNA-damaging species are formed at the level of the respiratory chain. It is important to note, however, that this is not necessarily true since it is well known that mitochondrial Ca^{2+} uptake is driven by the proton electrochemical gradient across the inner membrane[15,16] and this will increase only under conditions in which the NADH-linked substrates stimulate electron transport. Indeed, using U937 cells we found that pyruvate, under the same conditions in which it stimulates oxygen consumption, also promotes rotenone-sensitive mitochondrial Ca^{2+} accumulation.[9] Thus, electron transport is an upstream event allowing mitochondrial Ca^{2+} accumulation in cells supplemented with pyruvate. As a consequence, the possibility exists that the mechanism whereby the NADH-linked substrates enhance tha formation of tB-OOH-derived DNA-damaging species is Ca^{2+} -dependent and takes place in the mitochondria in a site that is distal to the respiratory chain, as observed with Cf or ATP. This possibility finds support in the experimental data that will be discussed below.

The first experimental evidence providing a link between AA and the increased formation of DNA lesions generated by tB-OOH in response to treatments resulting in increased mitochondrial Ca^{2+} accumulation was that three different PLA_2 inhibitors prevented the enhancing effects mediated by Cf, ATP or pyruvate. Indeed, very low concentrations of mepacrine (Figure 2A), BPB (Figure 2B) and ETYA (Figure 2C) suppressed the enhancing effects mediated by the Ca^{2+}-mobilizing agents and by the respiratory substrate. The fact that these inhibitors did not reduce the DNA cleavage generated by tB-OOH alone (Figure 2 A-C) or combined with antimycin A (Figure 2 A-C) rules out non-specific protection mechanisms and therefore strongly suggests that the effects of mepacrine, BPB and ETYA were specifically linked to their ability to inhibit PLA_2.

This conclusion is supported by the observation that the genotoxic response evoked by tB-OOH is enhanced by addition of reagent AA (Figure 4A) or by the PLA_2 activator melittin (Figure 4B). The enhancing effects of melittin, unlike those of AA, were prevented by mepacrine (Figure 4B). This finding on the one hand indicates that the effects of melittin are mediated by activation of PLA_2 and on the other hand further emphasizes the specificity of the inhibitory effects afforded by mepacrine.

These results suggest that the PLA_2 pathway is involved in the Cf-, ATP- and in the pyruvate-mediated enhancement of the tB-OOH-induced DNA cleavage in U937 cells. As a consequence, it would appear that in pyruvate-supplemented cells, as observed with Cf or ATP, the formation of tB-OOH-derived DNA-damaging species takes place in a site that is distal to the respiratory chain (see above). Interestingly, the addition of melittin or AA did not however further increase the accumulation of DNA lesions in cells exposed to tB-OOH and each of the Ca^{2+}-mobilizing agents or the respiratory substrate (not shown), strongly suggesting that melittin or AA on the one hand, and Cf, ATP or pyruvate on the other, act via common mechanisms, i.e. by activating the PLA_2 pathway.

Mitochondria are known to possess a Ca^{2+}-dependent PLA_2[17] which regulates the turnover of membrane phospholipids as well as the process of Ca^{2+} release from the mitochondria.[18] The experimental results described above, along with those previously obtained in our laboratory, are consistent with the possibility that the specific PLA_2 pathway involved in the Cf-, ATP- or pyruvate-mediated enhanced

formation of tB-OOH-derived DNA-damaging species is located at the level of mitochondria. This inference finds experimental support in a number of different observations. Indeed, our previous work clearly demonstrated that the enhancing effects of Cf,[7] ATP[8] and pyruvate[9,10] are apparent only under conditions of enforced Ca^{2+} accumulation in the mitochondria. The experimental approach utilized in this study allowed us to further discriminate between the effects of mitochondrial *vs* cytosolic Ca^{2+}. We report that a mepacrine- (or other PLA_2 inhibitor-) sensitive formation of DNA lesions occurred in the presence of Cf or ATP (which increase both the mitochondrial and cytosolic Ca^{2+}[7–8]) as well as in the presence of pyruvate (which increases the mitochondrial Ca^{2+} accumulation and does not cause detectable changes in the cytosolic levels of the cation).[9,10] This would suggest that an increase in $[Ca^{2+}]_i$ is dispensable for the enhanced formation of the tB-OOH-derived DNA-damaging species. In addition, formation of the mepacrine-sensitive DNA lesions in ATP-supplemented cells was blunted by a concentration of Ry preventing the efflux of Ca^{2+} from Ry-sensitive Ca^{2+} stores. Under these conditions, although the $[Ca^{2+}]_i$ was remarkably elevated, the cation was not cleared by the mitochondria.[8]This indicates that the cytosolic fraction of the cation does not promote activation of the specific PLA_2 pathway involved in the formation of the DNA-damaging species.

The experiments using RR and Ry to manipulate intracellular Ca^{2+} homeostasis allow further considerations. The fact that RR, unlike Ry, prevents the enhancement of the tB-OOH-induced genotoxic response mediated by AA or melittin indicates that, under these conditions, Ca^{2+} is not mobilized from Ry-sensitive Ca^{2+} stores. More importantly, these results imply an involvement of mitochondrial Ca^{2+} also downstream to PLA_2 activation. As a consequence, it is likely that in cells supplemented with the Ca^{2+}-mobilizing agents or the respiratory substrates, an increase in mitochondrial Ca^{2+} will promote activation of both mitochondrial PLA_2 and additional downstream pathways. Likely canditates are the lipoxygenases which are Ca^{2+}-requiring for their activation.[19]

Since the metabolism of arachidonic acid promotes the formation of an array of eicosanoids, it is likely that one, or more, of these either directly or indirectly promote DNA cleavage. Interestingly, 12-hydroxy or hydroperoxy-6, 8, 11, 14 eicosatetraenoic acids were reported to induce DNA single strand breakage in human lymphocytes.[20] Lipoxygenases are also known to promote formation of reactive oxygen species[19] which may then cause DNA strand scission.

All together our results can be summarized in the following model: the Ca^{2+} mobilized by tB-OOH is taken up by the mitochondria[7] and, under these conditions, the calcium-mobilizing agents Cf[7] or ATP[8]or the respiratory substrate pyruvate[9,10] promote additional mitochondrial accumulation of the cation. The increased accumulation of Ca^{2+} in the mitochondria is paralleled by, and causally-linked to, an enhanced formation of DNA lesions which appear to be mediated by the PLA_2 product AA. Although mitochondrial Ca^{2+} accumulation is an event upstream to PLA_2 activation, the cation also regulates downstream events leading to formation of the final DNA-damaging species. Likely candidates are the metabolites of the lipoxygenase pathway.

Acknowledgements

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